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*Minireview*

# NMR Studies of Retinal Proteins

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A review is given of the use of nuclear magnetic resonance (NMR) spectroscopy to study bacteriorhodopsin and bovine rhodopsin. Solution and solid-state approaches are included. The studies of the bacterial proton pump examine the chromophore, the peptide backbone, and the protein side chains. The studies of the bovine visual pigment are limited to the chromophore. Various forms of each pigment are considered. Both structural and dynamic features are addressed.

**KEY WORDS:** Review; NMR; bacteriorhodopsin; rhodopsin; retinal; opsin; chromophore; Schiff base.

## INTRODUCTION

Nuclear magnetic resonance (NMR) techniques developed during the past decade now permit the determination of the structure of small soluble proteins without crystallization (Clore and Gronenborn, 1991). For small molecules in solution, rapid rotational diffusion averages the anisotropic interactions of the nuclear spins to the isotropic values. This includes the interaction with the electron currents induced by the applied field (the chemical shift or shielding), the interactions with other nuclear spins, indirectly through bonds (the *J*-coupling) or directly through space (the dipolar interaction), and, for nuclear spins $\geqslant 1$, the interaction with the electric field gradient (the quadrupole interaction). Because of the motional averaging of the interactions, the spectra are well resolved and, since the isotropic values of the dipolar and quadrupolar interactions are zero, the features of the NMR spectra are dominated by the chemical shifts and the *J*-couplings. The influence of the dipolar interactions is seen only in spin relaxation, giving rise to the nuclear Overhauser effect (NOE) which is one of the main tools utilized in protein structure determination by solution NMR.

However, for many proteins, including the retinal pigments, solution NMR techniques are problematic. If the solubility is low, it may not be possible to dissolve sufficient protein to obtain adequate signal-to-noise. Even with enough protein, if the molecule is large, or embedded in a large complex, the rotational diffusion will be too slow to adequately average the anisotropy of the nuclear interactions. As a result, resolution is lost. And, since the anisotropy of the chemical shift and dipolar and quadrupolar interactions far exceeds that of the *J*-couplings, for sufficiently slow rotational diffusion the *J*-couplings become relatively insignificant. Thus "solid-state" NMR is phenomenologically distinct from "solution" NMR.

To simplify solid-state spectra, dipolar interactions can be removed by studying dilute spins (e.g., naturally occurring $^{31}P$ or specifically introduced $^{13}C$, $^{15}N$, or $^{2}H$) with high-power decoupling of the protons. In the decoupled spectra of dilute spins, the breadth and shape of the signal (i.e., the powder lineshape) reflects the rate and anisotropy of the motion of the group carrying the nucleus. Thus, the solid-state spectrum contains dynamic information, the details of which are usually inferred by comparisons with simulations.

If more than one group in the molecule is isotopically labeled, the powder patterns generally overlap and it is necessary to spin the sample about an axis tilted at 54°44' (the magic angle) from the applied field

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to resolve the signals. In this way, the powder patterns can be broken up into a series of sharp lines centered at the isotropic chemical shift and spaced at the spinning frequency. The intensities of these lines depend on the anisotropy of the interactions, and therefore provide additional information. Thus, for a spin 1/2 nucleus (i.e., with no quadrupole interaction, as for $^{13}C$, $^{15}N$, and $^{31}P$) in a large or insoluble protein, a combination of high-power proton decoupling and magic angle spinning (MAS) yields a high-resolution spectrum from which one can obtain all three principal values of the tensor describing the chemical shift interaction, instead of just the average of the three which corresponds to the isotropic chemical shift. This additional information provides a more detailed picture of variations in the electronic structure in the vicinity of the nucleus. Interpretation of chemical shift data is generally empirical, based on comparisons with model compound data (although theoretical analyses are gradually improving to the point where there is a prospect for them to become useful).

More structural information can be obtained from solid-state NMR studies by reinstating dipolar interactions in a controlled way. For example, if spins are physically present as dilute homonuclear pairs, rather than individually, the rate of magnetization exchange between the two, when the magic angle spinning speed is adjusted to match a submultiple of the isotropic shift separation (i.e., to make the spinning sidebands of the two signals coincide), gives a measure of the distance between them. This is the basis of the new technique known as rotational resonance (Raleigh *et al.*, 1988; Levitt *et al.*, 1990). A related technique, REDOR, is useful for measuring distances between heteronuclear spin pairs (Gullion and Schaefer, 1989a, b).

As will be described below, both solution and solid-state NMR techniques have been used to study retinal proteins. In the former case, it is necessary to solubilize the membrane. This raises questions about the functional state of the protein. Furthermore, even the solubilized complex is large and the resolution is poor. However, some suggestive results have been obtained on such preparations. Greater progress has been made using solid-state techniques. In the earliest solid-state investigations, the membranes were lyophilized. However, with the advent of improved rotors, capable of spinning somewhat fluid samples, most of the NMR studies have been done on wet membrane pellets, at temperatures ranging from ambient to well below freezing. These extensive studies have confirmed the few early results on lyophilized samples.

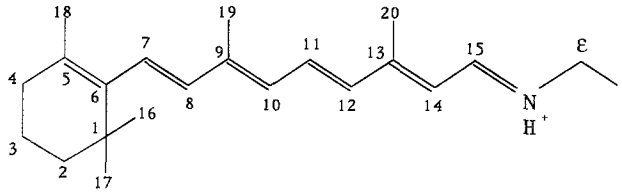


**Fig. 1.** Numbering scheme for retinal Schiff base-linked to a lysine residue.

The following review of NMR studies of retinal proteins is organized into sections focusing on the various parts of the molecules: the chromophore, the peptide backbone, and the protein sidechains. Since the chromophore has been the most thoroughly studied, that section is divided according to the various species that have been considered.

## NMR STUDIES OF THE RETINAL CHROMOPHORE

### Solubilized Bovine Rhodopsin (Rh) and Bacteriorhodopsin (bR)

In the late 1970's and early 1980's, Mateescu and co-workers published the first NMR studies of a rhodopsin (Shriver *et al.*, 1977, 1982). For [14-$^{13}C$] enriched 11-*cis*-retinal (see Fig. 1 for retinal numbering scheme) in Rh solubilized at a concentration of 0.5 mM in 0.1 M $KH_2PO_4$ buffer (pH 7.0) with 100 mM octyl glucoside, they found a resonance about 10 ppm downfield from the corresponding resonance for a protonated Schiff base (SB) of retinal, and very close to the resonance for an unprotonated SB of retinal or free retinal. Subsequently, Yamaguchi and co-workers (1981) reported results for bR solubilized in potassium phosphate buffer (pH 7.0) with 0.1 M octyl-$\beta$-D-glucoside. They found that for [14-$^{13}C$] retinal labeled bR there were two resonances, at 125 and 118 ppm relative to TMS. The resonance at 118 ppm was in the range expected for a protonated SB and the resonance at 125 ppm corresponded to the resonance observed by Mateescu and co-workers in their Rh preparation. For [15-$^{13}C$] retinal labeled bR, Yamaguchi *et al.* observed only one resonance. This resonance, at 191 ppm, was characteristic of free retinal. Based on these results, they suggested that Mateescu *et al.* had observed only free retinal in their Rh preparations. In further work, Mateescu *et al.* (1983, 1984)

studied [13-$^{13}$C]retinal labeled Rh and bR, solubilized at concentrations of 0.5 to 1.0 mM in octyl-$\beta$-glucoside/phosphate buffer (pH 7.0). In both the Rh and the bR, $^{13}$C-NMR evidence was obtained for a protonated SB.

### Dark-Adapted bR ($bR_{555}$ and $bR_{568}$)

Solid-state NMR studies of [$\varepsilon$-$^{15}$N]lys labeled bR in the dark-adapted state were reported independently by Mateescu *et al.* (1984) and by Harbison *et al.* (1983, 1984c); see also Smith *et al.* (1986a, b). The results showed that the Schiff base is protonated. With their better signal-to-noise and resolution, Harbison *et al.* found a doublet which was interpreted as being due to the coexistence of 13-*cis*- and *all-trans*-retinals in dark-adapted bR. They also noted that both chemical shifts in the bR doublet are still farther upfield from the signals of unprotonated model compounds than the corresponding chemical shifts of the halide salts of retinylidenebutyl[$^{15}$N]imine. This suggested that the SB in bR is more strongly protonated (i.e., more weakly hydrogen bonded) in bR than in the halide model compounds. Investigation of the three individual shift tensor elements in model compounds showed that the counterion for the protonated Schiff base affected the two downfield elements proportionately, without affecting the upfield element (de Groot *et al.*, 1989). For the *all-trans* component of dark-adapted bR it was found that the two downfield shift tensor elements conform to the same linear relationship found for the *all-trans* protonated SB model compounds. This suggested that a single dominant factor, namely the strength of the hydrogen bond between the SB and its counterion, might be the basis for the observed variation of the chemical shifts, including the extraordinarily upfield values in bR.

Harbison *et al.* (1988) also used $^{15}$N-NMR to study the exchange of the SB proton with bulk water. Since the relatively immobile protons of the protein relax much more rapidly than the protons in bulk water, delayed cross polarization of the nitrogen from protons will only be effective if protons near the nitrogen are exchanging rapidly with bulk water. For a delay of 1 ms, cross-polarization was observed for the SB and for the six free lysine residues, although there was no longer any cross-polarization of the natural abundance $^{15}$N in the peptide backbone. By varying the delay, it was demonstrated that proton exchange between the SB and bulk water is essentially complete in about 0.5 ms.

Protonation of the SB has been confirmed by $^{13}$C-NMR studies of bR specifically labeled at various positions in the delocalized $\pi$ system of the chromophore (Harbison *et al.*, 1984a, b; Smith *et al.*, 1986b). The $^{13}$C-NMR studies have also provided information on the conformation of the chromophore (Harbison *et al.*, 1984b, c; Smith *et al.*, 1986b) and its electrostatic environment (Harbison *et al.*, 1985; Smith *et al.*, 1986a, b; Mathies *et al.*, 1987a, b; Lugtenburg *et al.*, 1988). For most of the carbons in the polyene chain there is a doublet in the $^{13}$C spectrum due to the coexistence of 13-*cis* and *all-trans* chromophores in dark-adapted samples. The splitting is largest at the C-12 and C-14 positions (amounting to 10.1 and 11.5 ppm, respectively). The differences in the individual elements of the shift tensors are consistent with changes in a steric interaction between C-15 and C-12, related to isomerization of the $C_{13}$=$C_{14}$ bond, and a similar $\gamma$-effect between C-14 and the $\varepsilon$-carbon of Lys216, related to isomerization of the SB $C_{15}$=N bond. It was concluded that the 13-*cis* component of dark-adapted bR is also 15-*syn* and the *all-trans* component is 15-*anti*. This conclusion has recently been verified using rotational resonance measurements of the distances between C-14 on the retinal and the $\varepsilon$-carbon of Lys216, in both components of dark-adapted bR (Thompson *et al.*, 1992). The bicycle pedal isomerization between the two forms of the chromophore is presumably catalyzed or enforced by the surrounding protein.

$\gamma$-Effects are also important in distinguishing between the 6-*s*-*cis* and 6-*s*-*trans* conformations of the chromophore. Whereas the 6-*s*-*cis* conformation dominates in solution, the chemical shifts at C-5 and C-8 in bR are more consistent with a 6-*s*-*trans* conformation. The $T_1$'s of the C-18 (Harbison *et al.*, 1985) and C-16, 17 (Smith *et al.*, 1989b) methyl carbons are also more consistent with a 6-*s*-*trans* conformation in bR. Further support for the 6-*s*-*trans* conformation has been obtained from a $^2$H-NMR study of methyl group dynamics. Copié *et al.* (1990) found that motional narrowing in the $^2$H-NMR spectra of [18-$CD_3$]retinal bR is comparable to that seen in 6-*s*-*trans* retinoic acid, and greater than that seen in 6-*s*-*cis* retinoic acid. Finally, the 6-*s* conformation in bR has been examined by rotational resonance measurement of the distance between C-8 and C-18 in doubly $^{13}$C labeled samples (Creuzet *et al.*, 1991). The distance is

consistent with a 6-*s-trans*, rather than 6-*s-cis*, conformation. The evidence for isomerization of retinal from 6-*s-cis* to 6-*s-trans* on binding to the protein is important because lengthening the $\pi$ system contributes significantly to the large red shift in the visible spectrum of bR relative to a free retinal Schiff base.

Although the two upfield $^{13}C$ shift tensor elements at C-5 are in good agreement with the 6-*s-trans* model compound, the downfield element indicates another perturbation unrelated to 6-*s* isomerization (Harbison *et al.*, 1985). It was proposed that this might be due to a negative point charge in the binding pocket, if its positive counterion was suitably positioned to explain the absence of any effect on the chemical shifts of the other carbon atoms (Smith *et al.*, 1989b). However, it has since been suggested that the anomaly at C-5 might be a delocalized effect of the unusually weak hydrogen bonding of the SB at the other end of the polyene (Albeck *et al.*, 1992).

### Light-Adapted bR ($bR_{568}$)

In order to use NMR to study the effects of light on bR, it is necessary to stabilize the photoproducts for extended periods of time. This is accomplished most readily for the light-adapted state. The signals found in the $^{13}C$-NMR spectra of frozen, light-adapted bR corresponded to those assigned to the *all-trans* component of dark-adapted bR (Smith *et al.*, 1989b). These results supported the interpretations that had been made of the doublets at C-12 and C-14 in the dark-adapted spectra, in terms of $\gamma$-effects resulting from the isomerization of the $C_{13}=C_{14}$ and $C_{15}=N$ bonds.

### M Photointermediate of bR

In the M state, the SB has released, but not yet replaced, its proton. Therefore, this is a crucial intermediate in proton translocation. For $^{13}C$-NMR studies of the chromophore, the M intermediate has been thermally trapped at pH = 9.5–10 in 0.1 M NaCl or 0.5 M guanidine hydrochloride (Smith *et al.*, 1989a). Chemical shifts at C-12 and C-13 indicated that the M intermediate has a 13-*cis* chromophore with an unprotonated SB, in agreement with earlier resonance Raman results. The chemical shift at C-5 was very similar to that in dark-adapted bR, indicating a 6-*s-trans* structure and an additional electronic perturbation. At C-14, different chemical shifts were found in the two different M preparations. The results in guanidine hydrochloride indicated an *anti* C=N configuration, consistent with resonance Raman results. This interpretation is supported by a subsequent study of $\gamma$-effects at the $\varepsilon$-carbon of Lys216 (Farrar *et al.*, 1990). The results in NaCl were initially interpreted to indicate a C=N *syn* form of M, but difference spectroscopy has since shown that the M intermediate in NaCl is identical to that in guanidine HCl (McDermott *et al.*, 1991). The confusion was caused by the presence of a coexisting species.

### Low-pH Forms of bR ($bR_{600}$ and $bR_{565}$)

With decreasing pH the maximum in the visible absorption spectrum of bR shifts from 568 nm (pH = 7) to 600 nm (pH = 2), and then back to 565 nm (pH = 0). These purple-to-blue and blue-to-acid purple transitions were investigated by de Groot *et al.* (1990) using $^{13}C$ and $^{15}N$ NMR. The spectra of [$\varepsilon$-$^{15}N$]lysine labeled samples indicated that the $^{15}N$ chemical shift was closely correlated with the color. The 16 ppm upfield shift in $bR_{600}$ was interpreted to indicate an even weaker hydrogen bond to the counterion than that in $bR_{568}$ or $bR_{555}$. A complex counterion, involving an array of hydrogen bonds to diffuse the charge, was proposed to account for the $^{15}N$ spectra of the purple and blue species. $^{13}C$ NMR results for selectively labeled polyene carbons in $bR_{600}$ showed that the chromophore conformation is generally not significantly different from that in $bR_{568}$, although the spectrum of [14-$^{13}C$] retinal $bR_{600}$ exhibited four distinguishable subspecies.

### Rhodopsin, Isorhodopsin, and Bathorhodopsin

Bleached bovine Rh has been regenerated with selectively $^{13}C$ labeled retinals. Mollevanger *et al.* (1987) and Smith *et al.* (1987, 1990) have independently reported solid-state NMR results on Rh. To quench the slow rotational diffusion of the protein (which interferes with the cross polarization and decoupling required for good solid-state spectra), the former group lyophilized their lipid reconstituted samples while the latter froze their detergent solubilized samples. Spectra of [5-$^{13}C$] retinal labeled Rh indicated that in Rh the chromophore is 6-*s-cis* (Mollevanger *et al.*, 1987; Smith *et al.*, 1987). In addition, the [14-$^{13}C$] results of Smith *et al.* indicated that the SB of the chromophore in Rh is protonated and the C=N bond is *anti* (Smith *et al.*, 1987). Differences in chemical shift relative to the model compound were observed for positions 8 through 13, with the largest deviation at C-13 (Mollevanger *et al.*, 1987; Smith *et al.*, 1990).

This is consistent with models of the opsin shift in Rh that place a point charge near C-13. Results for isorhodopsin (Smith *et al.*, 1990) and bathorhodopsin (Smith *et al.*, 1991) also indicate a 6-*s*-*cis* chromophore and a perturbation near C-13 (Smith *et al.*, 1990).

## NMR STUDIES OF THE PEPTIDE BACKBONE

Because of the need for isotopic labeling, NMR studies of the protein have been restricted to bR.

Lewis *et al.* (1985) studied $^{13}C$ NMR powder spectra of [1-$^{13}C$]leu labeled bR in both intact purple membrane and DMPC vesicles. The spectrum of intact purple membrane showed a rigid-lattice carbonyl powder pattern which indicated that, at the leucine residues, the peptide backbone is immobile on the $^{13}C$ NMR time scale. In the vesicles, the spectrum was narrowed by fast rotational diffusion. Computer simulation of the motionally averaged line shape was used to analyze the possible orientations of the leucine peptide bonds relative to the diffusion axis.

Arseniev *et al.* have reported two-dimensional proton NMR studies of a synthetic 32-residue analog of segment B of bR (residues 34–65) (Arseniev *et al.*, 1988) and a synthetic 35-residue analog of segment D of bR (residues 102–136) (Maslennikov *et al.*, 1991) in the membrane mimetic system methanol/chloroform (1:1). They found that both peptides formed right-handed $\alpha$-helices with extremely rigid mid-regions, (i.e., from residue 42 to 60 in the B analog and from residue 107 to 131 in the D analog).

Deber *et al.* (1990) have studied [$\gamma$-$^{13}C$]pro labeled bR solubilized in $CHCl_3$:$CD_3OD$ (1:1) with 0.1 M $LiClO_4$. Sufficient resolution was achieved to distinguish the 2 ppm difference in $^{13}C$ chemical shift between a *cis* and a *trans* X-proline peptide bond. No signal was found corresponding to the *cis* conformation. It was concluded that in this preparation, all 11 prolines are in the *trans* conformation.

Bowers and Oldfield (1988) investigated the motion of the peptide backbone of bR by [1-$^{13}C$] enrichment of glycine, isoleucine, leucine, lysine, phenylalanine, and valine residues. In each case they found both mobile and immobile residues. Using integrated signal intensities they calculated that, except for valine, the numbers of highly mobile residues correlate with the numbers of residues in the C-terminus. They concluded therefore that the C-terminus is highly mobile on the $^{13}C$-NMR time scale. Since the C-terminus does not contain any valine residues, they suggested that the presence of mobile valine residues could indicate another mobile region of the protein. The discrepancy between their results for [1-$^{13}C$]leu bR and those of Lewis *et al.* (1985), described above, has not been resolved.

Helgerson *et al.* (1991) have also used [1-$^{13}C$] enrichment to study the bR backbone. Based on studies of homopolymers, in which the carbonyl resonance is found at 176 ppm for helical structures and at 171 ppm for nonhelical structures, they interpreted $^{13}C$ spectra of [1-$^{13}C$]leu bR, [1-$^{13}C$]val bR, and [1-$^{13}C$]lys bR to show that about 85% of the leucine, 75% of the valine, and 80% of the lysine residues in bR were in helical regions. These results are consistent with the current model of bR based on electron cryomicroscopy (Henderson *et al.*, 1990).

## NMR STUDIES OF PROTEIN SIDECHAINS

In the early 1980's, Oldfield and co-workers (Kinsey *et al.*, 1981a,b; Keniry *et al.*, 1984; Baianu *et al.*, 1984) reported a series of solid-state $^2H$-NMR studies of side-chain motions in bR. For deuterated gly, val, leu, ser, thr, lys, phe, tyr, and trp residues, the quadrupole echo spectra showed both a broad powder pattern and a sharp and intense central component. The latter was believed to be due to the surface residues in bR undergoing fast isotropic motion. On the other hand, they noted that once the C-terminal residues were removed, the intensities of the central components decreased dramatically. In the case of [$\gamma$-$^2H_6$]val bR, the drop was from 35% of the entire spectral intensity to just 7%. They therefore suggest that the amino acid side chains inside the membrane were immobile. Studies at 37°C showed that the three aromatic amino acids, tryptophan, tyrosine, and phenylalanine, displayed very different mobilities. Tryptophans in bR were rigid on the deuterium NMR time scale ($10^{-5}$s), but tyrosines and phenylalanines were undergoing rapid ($>10^{-5}$s) 2-fold ring flips. However, at 91°C all the aromatic residues underwent rapid motion and at $-30$°C all of the motion was frozen out.

Other $^2H$-NMR studies led to different results, Herzfeld *et al.* (1987) found for [$^2H$]leu labeled samples that the contamination of the purple membrane by residual plasma membrane (the "red membrane" in wild-type *Halobacterium halobium* but a colorless membrane in the carotenoid-free strains) contributed to the isotropic signal. For carefully purified purple

membrane, they found that few leucine residues in bR were moving fast on the $^2$H-NMR time scale. For [$^2H_5$]phe labeled bR, Griffin and co-workers (Rice *et al.*, 1981) found that even at $-30$°C, most of the phenylalanine rings (80%) were still executing 180° flips at a rate of about $10^3\,sec^{-1}$. At 12°C the rate increased to about $10^6\,sec^{-1}$ and at 25°C the hopping rate reached the fast limit (greater than $5 \times 10^7\,sec^{-1}$).

Engelhard and co-workers (Mayo *et al.*, 1988) used two-dimensional $^1$H-NMR to identify highly mobile aromatic residues in bR. For unlabeled samples solubilized in 0.1–1% (w/v) SDS/$D_2O$ they found that about three tyrosines, two tryptophans, and three or four phenylalanines were relatively mobile on the $^1$H-NMR scale ($> 10^3 s^{-1}$). Photochemically induced dynamic nuclear polarization experiments showed that only one tyrosine and one tryptophan is exposed to solvent.

Seigneuret *et al.* (1991) reported studies of [*methyl*-$^{13}$C]met labeled bR solubilized in Triton X-100. Using papain proteolysis and paramagnetic broadening, they showed that Met 68 is located at one of the hydrophilic surfaces of bR, and overall five out of nine methionines are located at the periphery of bR. Among these, one was assigned to the hydrophilic surface, three were assigned to the hydrophobic surface, and the last one was assigned to the boundary between these two surfaces. These results agree with the current model of bR structure based on electron cryo-microscopy (Henderson *et al.*, 1990). $T_2$ and NOE measurements suggested that of the nine methionines in the detergent-solubilized bR, six of them had a higher rate and amplitude of wobbling than the other three; but in the case of membrane-embedded bR only one methionine underwent fast wobbling.

Arseniev *et al.* (1987) has reported $^{19}$F-NMR studies of 5-fluorotryptophan labeled bR and its proteolytic fragments, solubilized in mixtures that retain the normal CD spectrum ($CD_3OH$–$CHCl_3$ (1:1), 0.1 M $LiCClO_4$, with or without 1.2 M HCOOH to protonated ionogenic groups). In the $^{19}$F-NMR spectrum of the intact protein, they observed six signals of equal intensity and one of double intensity, corresponding to the eight tryptophans in the protein. These signals were assigned to specific tryptophan residues by comparison with the $^{19}$F-NMR spectra of $NaBH_4$ and chymotryptic fragments. Increasing the level of fluorination, to detect possible interactions between the tryptophans, did not produce any change in the protein spectrum. However, changes in the spectrum were observed upon addition of HCOOH, upon reduction of the SB, and upon modification of the ionone ring of the chromophore, which indicated effects on different tryptophan residues in the three cases. Similar studies have been made of 3-fluorophenylalanine labeled bR and its fragments (Abdulaeva *et al.*, 1991). The signals in the spectra have been partially assigned and the interaction between Phe230 and a spin label introduced at Met163 indicates a distance of $13 \pm 1$ Å.

Beyond dynamics and folding, the amino acid side chains must play an important role in the movement of protons from the SB to the extracellular surface and from the intracellular surface to the SB. Amino acids capable of ionization or hydrogen bonding can participate in the proton pathway. Tyrosine and aspartic acid residues, in particular, have been implicated by FTIR studies.

Herzfeld and co-workers have $^{13}$C-labeled the 4' position of tyrosines in bR (Smith *et al.*, 1986a). Varying the pH from 2 to 12 and the temperature from $-20$ to $-90$°C, they found no evidence for long-lived tyrosinate in dark-adapted bR (Herzfeld *et al.*, 1990). Nor was evidence found for long-lived tyrosinate in thermally trapped light-adapted bR at pH 7 or 10, or in thermally trapped M at pH 10 (McDermott *et al.*, 1991). It was concluded that the changes in tyrosine residues detected by FTIR represented changes in hydrogen bonding.

Engelhard *et al.* (1989) have studied bR biosynthetically labeled with [4-$^{13}$C]asp. The carbonyl region of the $^{13}$C spectrum showed six resolved peaks superimposed on the broad natural abundance signal of the peptide backbone. The effects on the signal intensities of cross-polarization of the $^{13}$C from protons indicated that the two upfield signals were from protonated aspartic acids, presumably buried in the hydrophobic interior of the protein. Changes in the spectrum upon cleavage of the C-terminus suggested that normally sequestered aspartic acid residues become exposed to the aqueous environment. The spectrum observed for the blue form of the membrane is consistent with protonation of the water-accessible aspartate residues. Engelhard *et al.* (1990a) have also studied [4-$^{13}$C]asp enriched samples of the single-site bR mutants Asp96 → Asn, Asp96 → Gly, and Asp85 → Glu. From the spectra of the mutants, they concluded that the second most upfield resonance in the wild-type protein, belonging to one of two buried protonated residues, was the Asp96 signal. Further

studies with mutants (Engelhard *et al.*, 1990c) showed that substitution of Asp96 by Glu drastically affects the protein conformation, while substitution by Gln appears to have little effect.

Engelhard *et al.* (1990b) have also used solid-state NMR to study the arginine residues in bR. For [guanidinium-$^{13}$C]arg bR, [terminal-$^{15}$N]arg bR, and [$\delta$-$^{15}$N]arg bR, the solid-state NMR spectra showed two signals, one with the chemical shift expected for arginine and the other with the chemical shift expected for the peptide backbone. Delayed cross polarization experiments did not detect any rapid exchange of protons between the nitrogens and water. In contrast, Lakshmi *et al.* (1991) have observed three signals in the $^{15}$N spectra of [$\delta$-$^{15}$N]arg bR. They found two arginine signals, 5 ppm apart, in addition to the peptide signal. After 48 hours in $D_2O$ at neutral pH, the NMR studies showed that the peptide nitrogens remained protonated but the arginine residues were completely deuterated. At high pH, more rapid proton exchange was observed at some of the arginine residues, but not in the peptide backbone.

Farrar *et al.* (1991) have used $^{15}$N-NMR spectroscopy to study the proline and tryptophan residues in dark- and light-adapted bR. The spectrum of dark-adapted [$^{15}$N]pro bR showed that one of the 11 proline residues is particularly distinctive, and the other 10 divided into two approximately equally populated groups with a chemical shift difference of 3 ppm. The dark-adapted spectrum of [indole-$^{15}$N]trp bR is less well resolved, but small changes are detected on light adaptation.

## CONCLUSIONS

In general, the great strengths of NMR spectroscopy are its nonperturbing nature, its specificity, and its great versatility as a probe of both dynamics and structure. The weakness of NMR is the requirement of large amounts of well-labeled samples. For bacteriorhodopsin this has not been a great problem. For the bovine rhodopsins it has limited studies to the chromophores, and for other rhodopsins it has so far been totally prohibitive. However, with continuing progress in the expression of membrane proteins in unicellular organisms, the experience with bR should become ever more generalizable to other membrane proteins.

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