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Solid-State Nitrogen-15 Nuclear Magnetic Resonance Study of the Schiff Base in Bacteriorhodopsin†

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ABSTRACT: Solid-state ^{15}N NMR has been employed to examine protonation of the Schiff base linkage in ϵ-[^{15}N]lysylbacteriorhodopsin, the single protein in purple membrane. It is shown with spectra of model compounds that protonation of a Schiff base results in an approximate 150-ppm change in the isotropic ^{15}N chemical shift. Concurrently, the breadth of the shift anisotropy decreases by a factor of about two from 600 to 270 ppm. The isotropic shift of the Schiff base linkage observed in dark-adapted ϵ-[^{15}N]lysylbacteriorhodopsin closely matches those observed for the protonated model compounds, particularly the more weakly hydrogen-bonded ones. It also seems to be affected slightly by isomerization of the retinal.

Bacteriorhodopsin (bR),[1] the single protein of the purple membrane of *Halobacterium halobium*, possesses as its chromophore the polyene aldehyde, retinal, Schiff base linked to the ϵ-amino group of a lysine side chain (Schreckenbach et al., 1977). For some years, the consensus has been that the aforementioned Schiff base or imine is protonated in the ground states of the dark- and light-adapted forms of the protein (bR_{560} and bR_{570}, respectively) (Lewis et al., 1974; Aton et al., 1977). Deprotonation of the imine is thought to occur during the photocycle (Lewis et al., 1974; Terner et al., 1979). Support for this assertion has resided entirely in comparisons between vibrational spectra of the protein and those of simpler model imines and immonium salts, the bands of interest being selectively observed by resonance Raman spectroscopy (Mendelsohn, 1973; Lewis et al., 1974; Aton et al., 1977). Specifically, a band at 1640 cm^{-1}, which is strongly perturbed on isotopic replacement with ^2H (Oseroff & Callender, 1974; Lewis et al., 1974; Terner et al., 1979) or ^{15}N (Lewis et al., 1978; Argade et al., 1981), has been assigned to a C=NH^+ stretching mode and is said to correspond to the 1655-cm^{-1} absorption observed for this linkage in protonated retinylideneimines (Heyde et al., 1971; Blatz & Mohler, 1975; Mathies et al., 1977). This frequency difference between the protein chromophore and model compounds, amounting to 15 cm^{-1}, is rationalized by postulating a weak hydrogen bond between the immonium proton and an anionic side chain on the protein (Blatz & Mohler, 1975; Rothschild & Marrero, 1982). Other discrepancies in the fingerprint region of the Raman spectrum have been attributed to the influence of the protein environment on the vibrational modes of the chromophore, a hypothesis that is difficult to test experimentally. Interpretation of the Raman spectrum is further complicated by the possibility that the exciting light induces changes in the conformation of the chromophore (Favrot et al., 1979) and that the observed spectrum therefore does not represent the ground-state configuration. It is precisely this possibility that provided the impetus for recent Fourier transform infrared experiments on bR (Rothschild & Marrero, 1982; Bagley et al., 1982). Therefore, while the weight of evidence appears to suggest some form of immonium structure for bR_{560} and bR_{570}, it is nonetheless desirable to obtain an entirely independent and, if possible, unambiguous corroboration of this postulate.

It has been shown that high-resolution ^{15}N NMR provides a means to discriminate between unsaturated nitrogenous bases and their conjugate acids (Witanowski et al., 1981). To date, the various classes of compounds containing basic C=N

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[1] Abbreviations: bR, bacteriorhodopsin; CP, cross polarization; MASS, magic angle sample spinning; ^{15}N-RB, retinylidenebutyl[^{15}N]-imine; ^{15}N-RBH^+Cl^-, retinylidenebutyl[^{15}N]immonium chloride; PM, purple membrane.

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linkages studied by ^{15}N NMR uniformly show upfield shifts of 100 ppm or more on protonation; in particular, several types of immonium salts have been shown to resonate at frequencies 100–140 ppm upfield of their respective imines (Allen & Roberts, 1980; Botto & Roberts, 1979). The size of this protonation effect is rather large and is therefore easily distinguishable from other smaller perturbations. For example, changes in solvent and/or counterions lead to changes in the isotropic shift which are an order of magnitude smaller. Unfortunately, conventional solution ^{15}N NMR of membrane proteins is only possible if the rotational correlation times are rendered sufficiently short to attenuate severe line broadening from dipolar couplings and chemical shift anisotropies. This usually entails "solubilization" in detergents such as octyl glucoside or Triton X-100 (Dencher & Heyn, 1978). The consequences are reduced sensitivity due to dilution, considerable residual line width, and most importantly, a perturbation of the experimental system from the native state.

^{15}N NMR spectroscopy of solids has several advantages over solution methods. First, with solid-state techniques it is possible to obtain spectra of unperturbed membrane samples (Haberkorn et al., 1978). Second, it can provide not only the isotropic chemical shifts, $\bar{\sigma}$, which may accurately be determined from MASS spectra, but also the three principal values of the chemical shielding tensor (σ_{11}, σ_{22}, σ_{33}). These latter quantities are extracted either from the intensities of rotational side bands in MASS spectra taken at low spinning speeds (Herzfeld & Berger, 1980; Maricq & Waugh, 1979) or directly from a static powder spectrum (Mehring, 1976; Haeberlen, 1976). Finally, the sensitivity of the technique is augmented by its use of undiluted materials and by the availability of cross polarization (CP), which provides enhanced polarization in times short compared to the normal spin–lattice relaxation times (Pines et al., 1973).

^{15}N NMR in solids is, however, much less well developed than in solution. The only study of an unsaturated nitrogenous base–conjugate acid pair in the solid state was conducted with L-histidine, ^{15}N-labeled on the imidazolium ring (Munowitz et al., 1982a). By use of both MASS and static samples, this work demonstrated the expected upfield shift of the imidazole nitrogens on protonation. In addition, it showed that this shift was caused predominantly by changes in σ_{22} and σ_{33} of the shielding tensor, indicating a proportionally larger change in the chemical shift anisotropy than in the isotropic shift. Thus the shift anisotropy is potentially a useful probe of the protonation state of a ^{15}N-labeled group. Furthermore, it was observed that hydrogen-bonding differences between two forms of the solid amino acid led to a change of 6 ppm in $\bar{\sigma}$, giving rise to the prospect that the hypothesis of strong hydrogen bonding between the putative immonium group in bR and some other group in the protein might be directly investigated by NMR.

We have therefore undertaken an ^{15}N NMR study of ϵ-[^{15}N]lysine-labeled purple membrane, of *all-trans*-retinylidenebutyl[^{15}N]imine, and of several *all-trans*-retinylidenebutyl[^{15}N]immonium salts, in the solid state. The results reported here employ both static and rotating powder samples.

Materials and Methods

Butyl[^{15}N]ammonium chloride, prepared from potassium phthal[^{15}N]imide (Stohler Isotope Chemicals, Waltham, MA) by the method of Vaughan et al. (1955), was added to freshly distilled triethylene glycol containing a fivefold excess of pulverized NaOH and distilled in a stream of dry nitrogen at 80–90 °C. The butyl[^{15}N]amine was collected in a liquid N_2 cooled trap over a period of several hours. This was reacted in 25% excess with *all-trans*-retinal (Aldrich, Milwaukee, WI) in dry methanol at −20 °C. The reaction mixture was allowed to stand overnight in the dark at that temperature, over an excess of 3-Å molecular sieves. After evaporation at 0 °C, the semisolid residue was recrystallized from dry acetonitrile. The yellow-orange retinylidenebutyl[^{15}N]imine (^{15}N-RB) was characterized by ^{1}H and ^{13}C NMR. ^{15}N-RB thus produced was dissolved in anhydrous ether and cooled to −40 °C. Dry HCl was bubbled through this solution to precipitate the hydrochloride salt. After most of the ether was decanted off, the residue was evaporated at −40 °C. The ^{15}N-RBH$^+$Cl$^-$, an orange crystalline powder, was used without further purification. Other salts of ^{15}N-RB were produced in a similar manner.

ϵ-[^{15}N]Lys-bR was prepared as previously described (Argade et al., 1981). Briefly, *Halobacterium halobium* R_1 was grown in a synthetic medium similar to that of Gochnauer & Kushner (1969) in which ϵ-[^{15}N]lysine was substituted for the lysine normally present. Incorporation was checked by including a tracer of [6-^{3}H]lysine, and it was found that 95% of the radioactivity was present in the lysine peak upon amino acid analysis of the HCl hydrolysate. Furthermore, the specific activity of the lysine in bR was comparable to that for lysine in the medium, indicating little if any synthesis of unlabeled lysine.

Samples of ϵ-[^{15}N]Lys-bR for the MASS ^{15}N NMR experiments consisted of ~100 mg of the lyophilized membrane preparation, which was packed into an Andrew-Beam-type delrin rotor. Typically, spinning speeds were ~3.2 kHz. Solid-state NMR spectra were obtained on a home-built spectrometer with an ^{15}N operating frequency of 29.8 MHz. Rotating fields of about 10 and 100 G were applied at the ^{1}H and ^{15}N frequencies, respectively. Cross polarization times were varied from 0.5 to 3 ms. Spectra of the model compounds required 10^2–10^3 scans, depending on whether spinning or static samples were examined. The final ϵ-[^{15}N]Lys-bR spectrum discussed below required ~10^5 scans. Recycle delays were typically 1–2 s.

In this report we present spectra of dark-adapted, lyophilized ϵ-[^{15}N]Lys-bR. The reason for this is the experimental simplicity of obtaining a MASS spectrum of unilluminated, lyophilized material as opposed to a hydrated and/or illuminated sample. We do not believe this significantly affects our results since Korenstein & Hess (1977a,b) noted no change in the absorption spectrum on dehydration of dark-adapted PM films at high vacuum, in contrast to the changes seen in light-adapted PM films. This suggests that the chromophore in the dark-adapted membrane remains unperturbed on lyophilization.[2] In addition, we have observed static ^{15}N powder spectra of fully hydrated, dark-adapted ϵ-[^{15}N]Lys-bR, and the results are in semiquantitative agreement with spectra discussed here. In particular, we do not observe spectral intensity in the 300–600-ppm region, which would be present if the Schiff base were unprotonated (see below). The effect of hydration and illumination on the details of the ^{15}N spectra is currently under investigation.

Results and Discussion

As mentioned above, isotropic and anisotropic ^{15}N chemical shifts of imines are extremely sensitive to whether or not the

[2] The red-shifted maximum observed in the optical spectrum is partially explanable in terms of a structure involving an immonium ion. However, in order to completely account for the size of the shift, it is necessary to introduce a negative charge close to the β-ionone ring (Honig et al., 1979; Balogh-Nair et al., 1981).

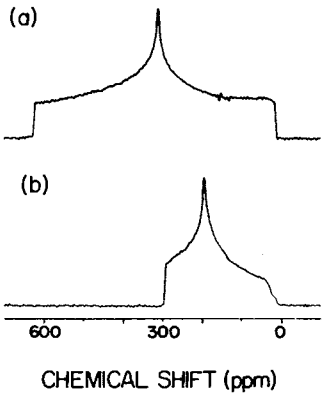

FIGURE 1: Proton-decoupled ^{15}N powder spectra of *all-trans*-retinylidenebutyl[^{15}N]imine: (a) unprotonated; (b) the HCl salt. In (a) the powder pattern is ~600 ppm (18 kHz) wide with an isotropic shift of 315 ppm. Protonation narrows the spectrum to a breadth of 270 ppm and moves the isotropic shift to 172 ppm.

Table I: Isotropic Shifts Derived from MASS (σ_I) and the Powder Pattern [(1/3) Tr($\tilde{\sigma}$)] and Chemical Shift Tensor Elements Obtained from Powder Spectra[a]

sample	σ_I	(1/3) Tr($\tilde{\sigma}$)	σ_{11}	σ_{22}	σ_{33}
^{15}N-RB	315.3	314.9	11.5	309.6	623.6
^{15}N-RBH$^+$Cl$^-$	171.7	172.5	27.3	194.6	296.3
^{15}N-RBH$^+$Br$^-$	166.1	165.5	26.7	183.3	286.6
^{15}N-RBH$^+$I$^-$	154.4	154.6	26.3	163.8	273.8
N-Lys-bR					
Schiff base	144.9 151.6	(av 148.3)			
amide backbone	93.6				
ϵ-Lys-$^{15}NH_3^+$	8.4				

[a] Estimated errors are ±1 ppm for the tensor elements and ±0.2 ppm for MASS isotropic shifts. All shifts are downfield from external 5.6 M $^{15}NH_4Cl$ in H_2O.

nitrogen is protonated. This is illustrated in Figure 1 with ^{15}N powder spectra of ^{15}N-RB and ^{15}N-RBH$^+$Cl$^-$. Figure 1a shows the spectrum of ^{15}N-RB, which exhibits an axially asymmetric powder pattern with a width of ~600 ppm. The principal values for this shift tensor are compiled in Table I, together with the isotropic shift, which is 314.9 ppm relative to external 5.6 M $^{15}NH_4Cl$. In ^{15}N-RBH$^+$Cl$^-$ the tensor is narrowed by more than a factor of two, to a breadth of about 270 ppm, as is shown in Figure 1b. Moreover, the isotropic shift moves to 172 ppm, a change of 142 ppm. Thus, protonation of the Schiff base linkage results in substantial changes in both σ and $\bar{\sigma}$. The effects are larger than are seen in, for example, ^{13}C spectra since the nitrogen is directly, rather than indirectly, involved in the protonation process. We should also emphasize that the changes in σ and $\bar{\sigma}$ associated with protonation of the Schiff base are about an order of magnitude larger than changes which are ascribable to solvent effects or counterions. For example, data are presented below which illustrate that changes in the counterion of ^{15}N-RBH$^+$ result in ~20-ppm alterations in the isotropic shift. The fact that these are clearly much smaller than those due to protonation permits us to unambiguously determine whether or not the Schiff base linkage in bR_{560} is protonated. It is also interesting that σ_{11} is relatively unchanged by protonation, whereas σ_{22} and σ_{33} move by about 120 and 330 ppm, respectively.

In Figure 2 are shown proton-decoupled ^{15}N MASS spectra of these two model compounds. The substantial differences in the isotropic and anisotropic shifts are again clearly apparent. At our operating field the ^{15}N frequency is 29.8 MHz, which, for a 600-ppm shift tensor, corresponds to a powder

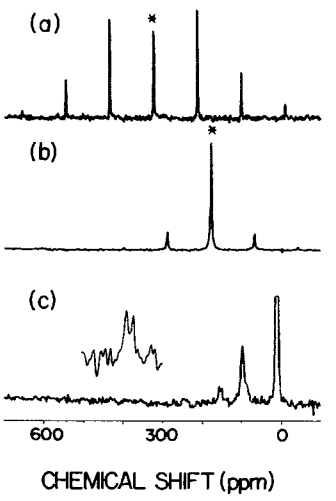

FIGURE 2: Proton-decoupled ^{15}N MASS spectra of (a) ^{15}N-RB, (b) ^{15}N-RBH$^+$Cl$^-$, and (c) ϵ-[^{15}N]Lys-bR. For clarity the center bands in the ^{15}N-RB and ^{15}N-RBH$^+$Cl$^-$ spectra are marked with asterisks. Note that the center band in the ^{15}N-RB spectrum is separated by 142 ppm from that of ^{15}N-RBH$^+$Cl$^-$ and the Schiff base line in the bR spectrum. The remaining two lines in the PM spectrum arise from the natural abundance peptide backbone nitrogens and the six lysine $-NH_3^+$ groups. The inset in (c) is an expansion of the Schiff base line showing the doublet structure. $\nu_R \simeq 3.2$ kHz.

pattern breadth of ~18 kHz. Thus, at a spinning speed of 3.2 kHz, the MASS spectrum exhibits a center band and three sets of rotational side bands. Note that because of the breadth of the tensor, the first set of side bands is slightly more intense than the center band. In contrast, the approximate 8-kHz powder pattern from ^{15}N-RBH$^+$Cl$^-$ is narrowed to a center band with a single set of weaker side bands.

Also shown in Figure 2 is the ^{15}N MASS spectrum of dark-adapted lyophilized ϵ-[^{15}N]Lys-bR. A comparison of this spectrum with those of the model compounds provides direct evidence that the Schiff base linkage in dark-adapted bR is indeed protonated. As is known from the amino acid composition of bR, there are seven lysines present in the protein (Khorana et al., 1979; Ovchinnikov et al., 1977, 1979). Six of these contain $-NH_3^+$ groups and give rise to the strong line at 8.4 ppm. It has been pointed out elsewhere that the shift anisotropy of $-^{15}NH_3^+$ groups is quite small—less than ~10 ppm (Harbison et al., 1981). As a result, there are no rotational side bands in the spectrum corresponding to this line. The line at 93.6 ppm arises from the natural abundance amide nitrogens in the peptide backbone. The shift anisotropy of such nitrogens is ~4.5 kHz at our field, and at 3.2-kHz spinning speeds, the majority of the intensity resides in the center band (Munowitz et al., 1982b). The remaining line in the bR MASS spectrum is a doublet centered at 148 ppm (the two components are at 145 and 151 ppm). As can be discerned from the figure, this line lies close to the center band of the ^{15}N-RBH$^+$Cl$^-$ spectrum and is removed from the ^{15}N-RB center band by ~150 ppm. Thus, the isotropic ^{15}N chemical shift is consistent with the presence of a protonated, rather than an unprotonated, Schiff base in lyophilized, dark-adapted PM. This interpretation is further supported by the fact that there are no rotational side bands present in the spectrum. If the Schiff base were unprotonated, then these side bands should be present with intensities approximately equal to that of the center band, as is illustrated in the ^{15}N-RB spectrum (Figure 2a).

There are three other features of the ϵ-[^{15}N]Lys-bR spectrum worth noting. First, as mentioned above, the Schiff base line is split into a doublet. The inset of Figure 2c, which is

an expansion of the Schiff base line, shows this doublet structure more clearly. Although the splitting is not much above the signal-to-noise ratio in the remainder of the spectrum, the splitting (and the chemical shifts) has been reproduced in multiple samples and spectra. For example, the full spectrum and inset of Figure 2c were obtained from different samples, and in both cases the splitting of the Schiff base line is apparent. The doublet structure therefore appears to be a real effect. It has been reported that dark-adapted bR contains a 1:1 mixture of 13-*cis*- and *all-trans*-retinal (Oesterhelt et al., 1973; Pettei et al., 1977). Since the absorption maximum of dark-adapted bR is unaffected by dehydration (Korenstein & Hess, 1977a,b), it is tempting to assign one line of the Schiff base doublet of our lyophilized sample to each isomer. That 13-*cis*- and *all-trans*-^{15}N-RBH$^+$Cl$^-$ exhibit different isotropic shifts seems to be borne out by the observation that a sample of 13-*cis*-^{15}N-RBH$^+$Cl$^-$ prepared in this laboratory exhibits a shift of 159 ppm, as opposed to 172 ppm observed for the all-trans compound. This shift difference, amounting to 13 ppm, is comparable to the 7-ppm splitting seen in the ϵ-[^{15}N]Lys-bR spectrum. Further investigations of this point are in progress.

Second, it is easily discerned from the spectrum of Figure 2c that the intensities of the lines are not in the theoretically expected 1:1:6 ratio. In particular, a single 90% ^{15}N-enriched site should display an intensity equal to the natural abundance peptide line. Correspondingly, the $-NH_3^+$ lysine line should contain 6 times the intensity of the natural abundance and Schiff base lines. In the experimental spectrum the integrated intensities are 1:2.5:7, when allowance is made for residual side-band intensities. Although we have considered several possible sources for this discrepancy, we do not at the moment have a satisfactory explanation for it. However, it is reasonably well-known that CP does often result in intensity anomalies. Thus, we do not consider this result surprising.

Finally, we noted above that the C=N Raman frequencies for the model compounds and the protein differ by 15 cm^{-1}. The same sort of differences appear in the ^{15}N NMR spectra, in that the shifts of ^{15}N-RBH$^+$Cl$^-$ and ϵ-[^{15}N]Lys-bR differ by 20 ppm. We have therefore examined other ^{15}N-RB salts, and results for the Br$^-$ and I$^-$ are shown in Table I. As can be seen from these data, both the isotropic and anisotropic shifts are qualitatively similar to those obtained from the Cl$^-$ salt. In particular, the isotropic shifts are close to those obtained for a protonated Schiff base, and the tensors are about 250 ppm wide. Nevertheless, the isotropic shift does decrease in the order Cl$^-$ > Br$^-$ > I$^-$, and the size of the tensor behaves in a similar fashion. The most obvious explanation of this trend is a reduction in hydrogen-bond strength between the immonium proton and anion, and thus in the NH bond polarization, as the anion size increases. In the absence of other factors, it may be expected that strongly hydrogen-bonded immonium residues should have ^{15}N shift parameters close to those for the chloride salt, and conversely, weakly bonded residues should tend toward those for ^{15}N-RBH$^+$I$^-$. The isotropic shift observed for ϵ-[^{15}N]Lys-bR is clearly in the latter category and thus suggests that this group is not strongly hydrogen bonded in bR.

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Registry No. RB, 36076-04-7; RB·HCl, 28448-64-8; 13-*cis*-RB·HCl, 69926-38-1; RB·HBr, 28448-68-2; RB·HI, 36076-08-1.

References

Allen, M., & Roberts, J. D. (1980) *J. Org. Chem. 45*, 130–135.

Argade, P. V., Rothschild, R. J., Kawamoto, A. H., Herzfeld, J., & Herlihy, W. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1643–1646.

Aton, B., Doukas, A. G., Callender, R. M., Becher, B., & Ebrey, T. G. (1977) *Biochemistry 16*, 2995–2999.

Bagley, K., Collinger, G., Eisenstein, L., Singh, A. K., & Zimanyi, L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4972–4976.

Balogh-Nair, V., Carriker, J. D., Honig, B., Kamat, V., Motto, M. G., Nakanishi, K., Sen, R., Sheves, M., Tanis, M. A., & Tsujimoto, K. (1981) *Photochem. Photobiol. 33*, 483–488.

Blatz, P., & Mohler, J. (1975) *Biochemistry 14*, 2304–2309.

Botto, R. E., & Roberts, J. D. (1979) *J. Org. Chem. 44*, 140–141.

Dencher, N., & Heyn, M. P. (1978) *FEBS Lett. 96*, 322–326.

Favrot, J., Leclercq, J. M., Roberge, R., Sandorfy, C., & Vocelle, D. (1979) *Photochem. Photobiol. 29*, 99–108.

Gochnauer, M. B., & Kushner, D. J. (1969) *Can. J. Microbiol. 15*, 1157–1165.

Haberkorn, R. A., Herzfeld, J., & Griffin, R. G. (1978) *J. Am. Chem. Soc. 100*, 1296–1298.

Haeberlen, U. (1976) in *High Resolution NMR in Solids: Selective Averaging*, pp 24–30, Academic Press, New York, San Francisco, and London.

Harbison, G. S., Herzfeld, J., & Griffin, R. G. (1981) *J. Am. Chem. Soc. 103*, 4752–4754.

Herzfeld, J., & Berger, A. E. (1980) *J. Chem. Phys. 73*, 6021–6030.

Heyde, M. E., Gill, D., Kilponen, R. G., & Rimai, L. (1971) *J. Am. Chem. Soc. 93*, 6776–6780.

Honig, B., Ebrey, T., Callender, R.-H., Dinur, U., & Ottolenghi, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2503–2507.

Khorana, H. G., Gerber, G. E., Herlihy, W. C., Gray, C. P., Anderegg, R. J., Nibei, K., & Biemann, K. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5046–5050.

Korenstein, R., & Hess, B. (1977a) *FEBS Lett. 82*, 7–11.

Korenstein, R., & Hess, B. (1977b) *Nature (London) 270*, 184–186.

Lewis, A., Spoonhower, J., Bogomolni, R. H., Lozier, R. H., & Stoeckenius, W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4462–4465.

Lewis, A., Marcus, M. A., Ehrenberg, B., & Crespi, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4642–4646.

Maricq, M. M., & Waugh, J. S. (1979) *J. Chem. Phys. 70*, 3300–3316.

Mathies, R., Freedman, T., & Stryer, L. (1977) *J. Mol. Biol. 109*, 367–372.

Mehring, M. (1976) *NMR: Basic Principles and Progress*, Vol. 11, pp 11–24, Springer-Verlag, New York.

Mendelsohn, R. (1973) *Nature (London) 243*, 22–24.

Munowitz, M., Bachovchin, W. W., Herzfeld, J., Dobson, C. M., & Griffin, R. G. (1982a) *J. Am. Chem. Soc. 104*, 1192–1196.

Munowitz, M., Aue, W. P., & Griffin, R. G. (1982b) *J. Chem. Phys. 77*, 1686–1689.

Oesterhelt, D., Meentzen, M., & Schumann, L. (1973) *Eur. J. Biochem. 40*, 453–463.

Oseroff, A. R., & Callender, R. H. (1974) *Biochemistry 13*, 4243–4248.

Ovchinnikov, Yu. A., Abdulaev, N. G., Fergina, M. Yu., Kiselev, A. V., & Lobanov, N. A. (1977) *FEBS Lett. 8A*, 1–4.
Ovchinnikov, Yu. A., Abdulaev, N. G., Fergina, M. Yu., Kiselev, A. V., & Lobanov, N. A. (1979) *FEBS Lett. 100*, 219–224.
Pettei, M. J., Yudd, A. P., Nakanishi, K., Henselman, R., & Stoeckenius, W. (1977) *Biochemistry 16*, 1955–1959.
Pines, A., Gibby, M. G., & Waugh, J. S. (1973) *J. Chem. Phys. 59*, 569–590.
Rothschild, K. J., & Marrero, H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4045–4049.
Schreckenbach, T., Walckhoff, B., & Oesterhelt, D. (1977) *Eur. J. Biochem. 76*, 499–511.
Terner, J., Hsieh, C. L., Burns, A. R., & El-Sayed, M. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3046.
Vaughan, W. R., Andersen, M. V., Blanchard, H. S., McCane, D. I., & Meyer, W. L. (1955) *J. Org. Chem. 20*, 819–822.
Witanowski, M., Stefaniak, L., & Webb, G. A. (1981) *Annu. Rep. NMR Spectrosc. 11B*, 33–34.

Articles

Constraints on the Flexibility of Bacteriorhodopsin's Carboxyl-Terminal Tail at the Purple Membrane Surface†

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ABSTRACT: A fluorescent probe, dansylhydrazine, was coupled to the purple membrane with a water-soluble carbodiimide. Fluorescence spectroscopy before and after proteolysis with papain indicated that the label was attached to the carboxyl-terminal tail of bacteriorhodopsin (residues 232–248). Reaction with [^{3}H]dansylhydrazine showed incorporation of 0.7 mol/mol of bacteriorhodopsin in this region. A minor site (0.3 mol/mol) was also labeled in the reaction, but its fluorescence was almost completely quenched by energy transfer to retinal. The conformation and flexibility of the C-terminal tail was studied by proteolysis and fluorescence polarization. Papain removes the C-terminal tail from the purple membrane in two steps, suggesting a segmented structure. At pH 8, 25 °C, the first-order rate constants were $k_1 = 0.23$ min^{-1} and $k_2 = 0.011$ min^{-1} for unmodified membrane. The cleavage mechanism was found to be sequential removal of the two tail segments. The labeled membrane had a similar mechanism but the first step was much slower: $k_1 = 0.026$ min^{-1} and $k_2 = 0.009$ min^{-1}. The steady-state polarization of the dansyl fluorescence on the tail was 0.24 at 25 °C, indicating a rigid environment. During proteolysis, the polarization decreased to 0.10 after 4 h. The time course of polarization decrease during proteolysis closely matched the rate of release of the inner tail segment. A model was derived for the polarization change during papain cleavage, on the basis of the assumption of only two tail states: covalently bound to bacteriorhodopsin and free in solution. This model gave a good fit to the proteolysis kinetics. Thus, the release of the outer segment does not affect the polarization, and hence the flexibility, of the inner segment. Time-resolved fluorescence anisotropy indicated no tail motion over two excited-state lifetimes. We conclude that the labeled region of the C-terminal tail of bacteriorhodopsin is rigidly held at the membrane surface.

Progress in diffraction and spectroscopic methods will provide increasingly detailed information about transmembrane proteins. However, the membrane surfaces are often the sites where membrane-mediated processes begin and end. Therefore, structural information must also be obtained about the surface regions of membrane proteins. So far, two approaches have provided information about membrane protein surfaces. Large extramembrane functional domains, of proteins such as cytochrome b_5, have been studied after removal from the membrane by proteolysis (Mathews et al., 1972; Spatz & Strittmatter, 1971). Exposed surface regions of proteins such as bacteriorhodopsin have been identified by proteolysis (Ovchinnikov et al., 1979) and chemical modification (Henderson et al., 1978; Dumont et al., 1981). Yet, little is known about the behavior of small surface segments of membrane-bound proteins. Undoubtedly, some membrane proteins will be found to utilize short surface segments in mediating effects between the surface and interior or between two surface sites on the same side of the membrane. We would like to know whether a membrane surface can provide special constraints on the folding of surface regions. In particular, are the amino and carboxyl termini (which are usually found to extend beyond the membrane surface) free to assume many conformations, or is their motion restricted in some way? The purple membrane is a useful model system for studying the dynamics of surface segments of membrane proteins. It contains only a single protein, bacteriorhodopsin, which functions as a light-induced proton pump [for a recent review, see Stoeckenius (1980)]. The amino acid sequence (Ovchinnikov et al., 1979; Khorana et al., 1979) and low-resolution crystal structure (Henderson & Unwin, 1975) have been determined. Although about 80% of bacteriorhodopsin is buried within the lipid bilayer, approximately 20 amino acids at the carboxyl

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