CONTRASTING MOLECULAR DYNAMICS IN RED AND PURPLE MEMBRANE FRACTIONS OF THE *HALOBACTERIUM HALOBIUM*

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ABSTRACT ^{2}H-nuclear magnetic resonance (NMR) has been used to study the dynamics of amino acid residues in bacteriorhodopsin with results that depend on the method of sample preparation. We show here that in [^{2}H]-leucine-labeled samples the intensity of the isotropic signal varies according to the degree of residual contamination of the sample with red membrane. We conclude that few of the surface leucine residues of bacteriorhodopsin are moving isotropically on the ^{2}H-NMR time scale.

INTRODUCTION

Solid-state ^{2}H-nuclear magnetic resonance (NMR) has been employed extensively to study molecular dynamics in macromolecules and supramolecular assemblies. Early application to membranes concentrated on lipid motion (for reviews see Seelig, 1977; Smith and Oldfield, 1984). More recently, ^{2}H-NMR has been used to examine the dynamics of amino acid residues in the purple membrane fragments of the *Halobacterium halobium* (Kinsey et al., 1981*a, b;* Rice et al., 1981; Rice, D. M., B. A. Lewis, S. K. DasGupta, J. Herzfeld, and R. G. Griffin, manuscript in preparation; Keniry et al., 1984*a, b*). These membrane fragments are thought to contain the single protein bacteriorhodopsin, which functions as a light-driven proton pump. Interestingly, different ^{2}H-NMR spectra have been observed with different preparations. For example, when we isolate Phe-d_5-labeled purple membranes according to the Oesterhelt and Stoeckenius (OS) procedure (1973), the spectrum shows only a broad $\eta \sim 0.6$ powder pattern Rice et al., 1981; Rice, D. M., B. A. Lewis, S. K. DasGupta, J. Herzfeld, and R. G. Griffin, manuscript in preparation). This indicates that essentially all the Phe-d_5 groups experience only anisotropic motion on the ^{2}H-NMR time scale. In contrast, published spectra of Phe-d_5-labeled purple membranes isolated according to an abbreviated version of the Becher and Cassim (BC) protocol (1975), exhibit a narrow line superimposed on the broad powder pattern (Kinsey et al., 1981*b*; Keniry et al., 1984*a*). This indicates that these preparations have a population of labeled groups undergoing isotropic or pseudo-isotropic motion on the ^{2}H-NMR time scale. Based on the relative intensities of the narrow and broad components in the spectrum, Keniry et al. (1984*a*) have attributed the narrow signals to residues on the bacteriorhodopsin surface and inferred that these groups are more mobile than those in the interior of the membrane.

In exploring the discrepant NMR results, we have repeatedly found that preparation of purple membranes according to the abbreviated BC method results in ^{2}H spectra similar to those published by Keniry et al. (1984*a*). It is possible to interpret these results as indicating greater proteolysis and/or aggregation of the purple membranes in the samples prepared by the OS method than in the samples prepared by the abbreviated BC procedure. However, when using gel electrophoresis we find no evidence of proteolysis in our samples, and when using electron microscopy we have found no correlation between membrane aggregation and the characteristics of the ^{2}H spectrum. Thus there must be some other important difference between the preparations.

In the description of their protocol, Becher and Cassim (1975) noted that a sucrose gradient is necessary at the end to remove the ⅓–⅔ of the purple membrane that still has significant amounts of the red plasma membrane attached. Since the abbreviated protocol used by Keniry et al. (1984*a*) omitted this step (Kunwar, A. C., private communication), we surmised that their preparations contain significant amounts of red membrane and that this might give rise to the narrow ^{2}H signal that is absent in OS preparations. We show here, in Leu-d_3-labeled samples, that the degree of residual contamination of the sample with red membrane depends on the method of sample preparation and correlates with the intensity of the iso-

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tropic ^{2}H-NMR signal. Thus, it is not necessary to invoke pseudo-isotropic motion of surface residues to explain the isotropic component of the ^{2}H-NMR spectrum, or membrane aggregation to explain the effects of trypsin treatment on the ^{2}H-NMR spectrum. These results suggest that other studies of bacteriorhodopsin dynamics (e.g., ^{13}C-NMR, ^{15}N-NMR, and fluorescence) be reviewed for possible artifacts due to residual red membrane components.

In addition, we discuss some difficulties in the interpretation of intensities in ^{2}H quadrupole echo spectra. It has been predicted theoretically (Spiess and Sillescu, 1981; Wittebort et al., 1987) and confirmed experimentally (Rice et al., 1987; Rice, D. M., E. T. Olejniczak, S. K. DasGupta, J. Herzfeld, and R. G. Griffin, manuscript in preparation; Beshah et al., 1987; Pschorn and Spiess, 1981) that a large fraction of the spectral intensity is lost for groups undergoing intermediate rate motion. As a result, it is not generally possible to integrate the components in an ^{2}H quadrupole echo spectrum and associate these with the number of spins of a given type.

METHODS

The JW-3 strain of *Halobacterium halobium* was grown on a synthetic medium like that of Gochnauer and Kushner (1969) except that the D-amino acids and the NH_4Cl were omitted, and 0.2 g/l 5-^{2}H,L-leucine was substituted for the usual 0.8 g/l unlabeled leucine. Purple membranes (PM) are usually prepared according to the OS procedure. For comparison, membranes were also prepared according to the abbreviated BC protocol (PM-1) and then washed further to reduce the amount of red membrane (PM-2). The specific procedures for these two preparations were as follows.

PM-1: Cells from a 4-liter culture were suspended in 400 ml of ice-cold water and placed into a blender for 10–15 min, during which 2–5-s bursts every 30–45 s were used to break up the DNA released by cell lysis. The volume of the suspension was then brought to 960 ml and stirred for 1 h at 4°C. The membranes were collected by centrifugation at 23,500 *g* for 2 h at 4°C, and then washed by stirring in 176 ml of ice-cold water for 1 h at 4°C and centrifuging at 100,000 *g* for 1 h at 4°C. This wash was repeated twice more (i.e., until the supernatant was colorless). The sample was then washed three times with ^{2}H depleted water by resuspending in a volume at least ten times that of the pellet and spinning at 245,000 *g* for 60 min.

PM-2: The sample prepared as above was stirred in 400 ml of cold 0.1 M NaCl at 4°C overnight and centrifuged at 39,000 *g* for 40 min at 4°C. The membranes were then washed three times with cold 0.1 M NaCl and three times with cold water by resuspending with a tissue grinder and centrifuging at 39,000 *g* for 40 min at 4°C. The sample was finally washed three times with ^{2}H-depleted water by resuspending in a volume at least ten times that of the pellet and spinning at 245,000 *g* for 60 min.

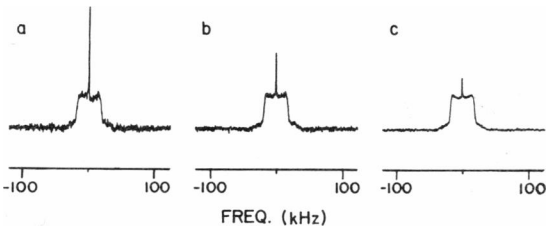

FIGURE 1. 61.4 MHz ^{2}H quadrupole echo spectra of Leu-d_3-PM. (*a*) PM-1 prepared according to the abbreviated BC procedure employed by Keniry et al., (*b*) PM-2 prepared as described in the text, and (*c*) PM prepared according to the OS procedure.

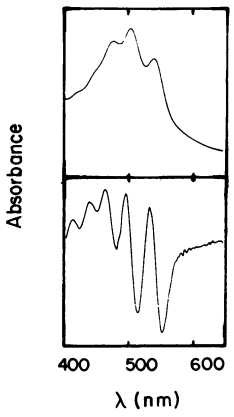

FIGURE 2. The visible absorption spectrum of RM (*top*) and the corresponding first derivative spectrum (*bottom*). Note the three peaks characteristic of carotenoids.

Red membranes (RM) were obtained from the supernatants of the first few washes of the purple membrane isolation. These were collected by spinning at 245,000 *g* for 60 min, and washed three times with ^{2}H-depleted water by resuspending in a volume at least ten times that of the pellet and spinning at 245,000 *g* for 180 min.

Intact membrane samples were examined for carotenoid content by visible spectroscopy using a diode array spectrophotometer (model 8451A; Hewlett-Packard Co., Palo Alto, CA). ^{2}H-NMR spectra were obtained with a quadrupole echo sequence (Davis et al., 1976) on a home-built spectrometer operating at 61.4 MHz for ^{2}H. Echos were digitized at 1 μs/pt, and 2,048 data points were collected before Fourier transformation. The spectra shown here have 200 Hz linebroadening applied to them.

RESULTS

Fig. 1 shows the ^{2}H-NMR spectra obtained for the three purple membrane samples. The spectrum obtained for PM-1 reproduces that reported by Keniry et al. (1984*a*) for this preparation. The spectra for PM-2 and PM exhibit a progressively smaller central spike.

Figs. 2–5 show the visible absorption spectra obtained for RM, PM-1, PM-2, and PM. The RM spectrum corresponds closely with that of Oesterhelt and Stoeckenius (1973) and shows the three peaks between 470 and 560 nm characteristic of the carotenoids. These peaks are seen in the PM-1 sample, to a reduced extent in the PM-2 sample, and to a still lesser extent in the PM sample. (A separate unlabeled sample, prepared the same way as the

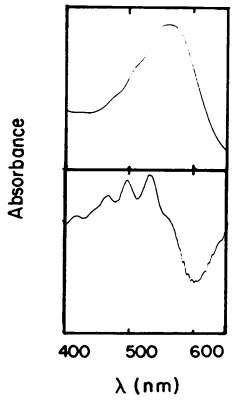

FIGURE 3. The visible absorption spectrum of PM-1 prepared according to the abbreviated BC procedure used by Keniry et al. (*top*) and the corresponding first derivative spectrum (*bottom*). Note the clear presence of the RM-associated carotenoid peaks in this spectrum.

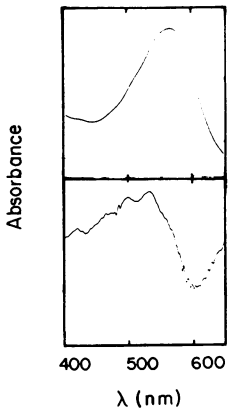

FIGURE 4. The visible absorption spectrum of PM-2 (*top*) and the corresponding first derivative spectrum (*bottom*). The lines associated with RM have decreased in intensity.

isotopically labeled PM-1, gave a similar spectrum to that shown here for the isotopically labeled PM-1.)

DISCUSSION

The visible spectra (especially the first derivative spectra) clearly show the presence of carotenoids in purple membrane samples. This contamination varies with the method of sample preparation and is correlated with the intensity of the isotropic component in the ^{2}H-NMR spectrum. It would therefore appear that most, if not all, of the mobile component seen in NMR spectra of purple membranes is due to residual non-bacteriorhodopsin proteins that accompany the residual carotenoids. Thus few if any of the surface residues in bacteriorhodopsin are moving isotropically on the ^{2}H-NMR time scale. This seems reasonable because, even in the surface regions linking transmembrane helices and in the functionally important COOH-terminal region, the protein structure would be expected to be quite constrained.

The loss of the isotropic NMR signal on proteolysis (Bowers, J. L., private communication) is best explained by preferential degradation of the contaminating protein. Since bacteriorhodopsin has been shown by electron microscopy to form a lattice in the membrane that excludes other proteins, the contaminants that we have observed are probably on the periphery of the membrane fragments. For this reason, and because it has a relatively large mobile component, the contaminant is probably more vulnerable to proteolysis than is bacteriorhodopsin. This would explain why the loss of the mobile NMR signal is greater than can be explained by the loss of bacteriorhodopsin surface residues. It is not necessary to suppose that there is any aggregation-linked immobilization of residues in proteolyzed samples.

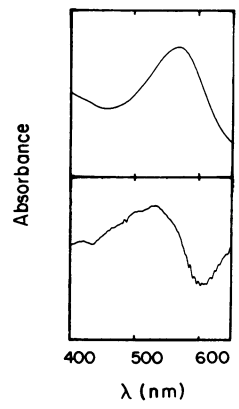

FIGURE 5. The visible absorption spectrum of PM prepared according to the OS procedure (*top*) and the corresponding first derivative spectrum (*bottom*). The RM lines are almost completely absent from the spectrum.

The mobile signal in the ^{2}H-NMR spectrum of PM-1 represents only a few percent of the total intensity in the spectrum. Although, as is discussed below, this intensity cannot generally be taken as a quantitative measure of the number of mobile groups, it is qualitatively clear that a pronounced mobile signal can result from a small population of mobile sites. If, in addition, we suppose that this small population is heterogeneous, representing several different plasma membrane proteins, then the amount of any one of these proteins could easily be 100-fold lower than the amount of bacteriorhodopsin. Such an amount would not be readily detected in gel electrophoresis. Under these circumstances, the carotenoid signature in the visible spectrum offers a more sensitive measure of purple membrane purity. In carotenoid-deficient mutants of *Halobacteria,* where this measure is not available, the purity of purple membrane preparations cannot be so readily monitored.

Finally, a few remarks about intensities in ^{2}H-NMR spectra obtained with quadrupole echo techniques are appropriate. Even though quadrupole echo sequences are commonly used to obtain ^{2}H-NMR spectra, it is not widely appreciated that the intensities in such spectra are not proportional to the number of spins except in the slow and fast limit motional regimes (Spiess and Sillescu, 1981; Wittebort et al., 1987). In particular, in the intermediate exchange region, T_2 becomes comparable to τ in the echo sequence and the second pulse does not refocus all of the magnetization. The intensity loss can be substantial and depends on the rate and mechanism of the molecular motion. For example, for ^{2}H's on an aromatic ring performing twofold 180° flips, only ~20% of the spectral intensity is observed when the flipping rate is $\sim 10^5$ s^{-1} (Wittebort et al., 1987; Rice et al. 1987; Rice, D. M., E. T. Olejniczak, S. K. DasGupta, J. Herzfeld, and R. G. Griffin, manuscript in preparation). For ^{2}H's involved in more symmetric motion the loss is still larger: for three- or four-site tetrahedral hops with approximately equal populations, the calculated intensity decreases to ~5% and ~0%, respectively, in the intermediate exchange regime ($\sim 10^5$ s^{-1}). The theoretical predictions have been confirmed experimentally with observations of the threefold motion of the CD_3 group in Ala-d_3 (Beshah et al., 1987) and the fourfold motion in hexamethylenetetramine (Pschorn and Spiess, 1980), where a relatively narrow line of much reduced intensity is observed. Thus, unless it can be determined that sidechains in a protein are executing fast or slow limit motion, it is incorrect to simply integrate the intensities to obtain the ratios of "mobile" vs. "immobile" residues. In bacteriorhodopsin, we have determined

that the minimum in the intensity of the Phe-d_4 spectrum due to twofold ring flips occurs at ~4°C, where ~25% of the intensity is observed (Rice, D. M., B. A. Lewis, S. K. DasGupta, J. Herzfeld, and R. G. Griffin, manuscript in preparation). This coincides with the temperature for the Phe-d_4 spectrum of Keniry et al. (1984*a*). Thus the intensity in their broad powder pattern is suppressed by about a factor of four. The intensity of their narrow line may also be suppressed by some undetermined factor and the ratio of mobile and immobile phenylalanine residues is unknown.

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