LIPID BILAYER DYNAMICS AND RHODOPSIN-LIPID INTERACTIONS: NEW APPROACH USING HIGH-RESOLUTION SOLID-STATE $^{13}$C NMR

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SUMMARY: High-resolution, solid-state $^{13}$C NMR spectra have been obtained for unsonicated multilamellar dispersions of 1,2-dilauryl-sn-glycero-3-phospho-choline (DLPC), recombinant membranes containing DLPC and rhodopsin, and native retinal rod disk membranes. The roles of $^1$H dipolar decoupling, $^1$H-$^1$C cross-polarization, and magic-angle sample spinning have been investigated. Rotating-frame $^{13}$C relaxation times have been measured and are discussed in terms of lipid bilayer dynamics and rhodopsin-lipid interactions.

It has become clear that the function of at least some membrane proteins depends on their lipid environment (1). To understand the effects of lipids on membrane protein function, one must physically characterize the molecular organization and dynamics of membranes. Nuclear magnetic resonance (NMR) spectroscopy is potentially one of the most useful tools for this purpose; however, previous $^{13}$C NMR studies have been hampered by the fact that membranes are large supramolecular structures which undergo relatively slow macroscopic reorientation, leading to unaveraged dipolar and chemical shielding anisotropy interactions. In this work, the effects of solid-state NMR techniques on the resolution of membrane $^{13}$C NMR spectra are examined. We have obtained high-resolution $^{13}$C NMR spectra of unsonicated multilamellar phospholipid suspensions and recombinant membranes containing a transmembrane protein, rhodopsin, as well as the first high-resolution $^{13}$C NMR spectrum of unsonicated native ROS.

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Abbreviations: DLPC, 1,2-dilauryl-sn-glycero-3-phosphocholine; ROS, rod outer segment.

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retinal rod disk membranes. In addition, we have performed $^{13}$C rotating-frame $T_1$ relaxation studies which provide information regarding the molecular dynamics of membraneous phospholipids as well as the nature of their interaction with rhodopsin.

**MATERIALS AND METHODS**

1,2-dilauryl-sn-glycero-3-phosphocholine (DLPC) was synthesized as described (2). Multilamellar dispersions of DLPC were prepared by adding an equivalent weight of doubly distilled H$_2$O and vortexing the sample until all the lipid was hydrated. Bovine retinas were obtained locally and rod outer segment (ROS) membranes prepared (3). The samples had $A_{280}/A_{500}$ absorbance ratios $\approx 2.8$ before regeneration, were $<15\%$ bleached, and were $>93\%$ regenerable (4). Rhodopsin was purified and reconstituted with DLPC (5) followed by suspension in 67 mM sodium phosphate buffer, pH 7.0. The sample had a lipid/protein ratio of 66:1 (mol/mol) and was homogeneous as judged by the appearance of a single, sharp band after continuous sucrose density gradient centrifugation. The DLPC/rhodopsin recombinant membranes contained $\approx 75\%$ wt% H$_2$O and were regenerable (6) before and after spectroscopy.

$^{13}$C NMR spectra were obtained at 50 MHz with a homebuilt NMR spectrometer capable of high-power $^1$H decoupling, $^1$H-$^{13}$C cross polarization, and magic angle sample spinning (MASS). All samples were freeze-thawed before spectroscopy and all spectra were recorded at ambient temperature. $T_1$ relaxation times were measured by the inversion recovery technique (7). The time constants for the decay of spin-locked $^{13}$C polarization, $T_1\rho$, were measured as described elsewhere (8). Relaxation times were obtained from single exponential fits of the data.

**RESULTS AND DISCUSSION**

Figure 1a shows a natural abundance $^{13}$C NMR spectrum of a 50 wt% multilamellar dispersion of DLPC in H$_2$O, obtained using continuous, low power $^1$H decoupling together with sample rotation at 2.4 kHz at the "magic-angle" (54.7°) with respect to the static magnetic field. Spectral assignments are given in the figure. The observed resolution equals or exceeds that in conventional Fourier transform (FT) $^{13}$C NMR spectra of small unilamellar vesicles (9). If, in addition to magic-angle sample spinning, high-power $^1$H dipolar decoupling is employed to obtain the $^{13}$C NMR spectra (8), little or no improvement in resolution or signal-to-noise results. Thus, for the case of lipid bilayers in the liquid crystalline (L$\alpha$) phase, spinning removes both residual static $^1$H-$^{13}$C dipolar interactions and $^{13}$C chemical shift anisotropies. For solid polymers (8) and bilayers in the gel phase (10), both dipolar decoupling and spinning are necessary to obtain high-resolution spectra.

Figure 1b shows a $^{13}$C NMR spectrum of a ca. 75 wt% aqueous dispersion of recombinant membranes containing DLPC and rhodopsin at a 66:1 lipid/protein
mole ratio, which closely approximates that of the native ROS disk membrane. The presence of rhodopsin is associated with a slight broadening of the lipid resonances, relative to the spectrum in Fig. 1a. Figure 1c shows a $^{13}$C NMR spectrum of native retinal ROS disk membranes. The observed resonances are due to the saturated, unsaturated, and polyunsaturated fatty acyl chains, the glycerol backbone, and the $^{+}$NM$e_3$ and other polar head-group carbons of the ROS membrane phospholipids (11,12). Resonances from rhodopsin were not observed,
either under the conditions of Figure 1, or with both dipolar decoupling and sample spinning.

For solids or solid-like samples many line broadening mechanisms are possible which are not present in liquid-state NMR (13). When both the spatial and spin-variables of the dipolar Hamiltonian are modulated, interference effects can occur (14). One possible explanation for the absence of rhodopsin lines in Figures 1b-c is that the known, relatively slow motions of the protein affect the coherent $^1$H-$^1$C dipolar decoupling. That is, rotational diffusion of rhodopsin with $\tau_R \approx 20$ ms (15) could lead to random modulation of the $^1$H-$^1$C dipolar interactions at frequencies comparable to $1/\tau_R \sim 50$ kHz, thereby defeating standard dipolar decoupling. Although further work is necessary, the above interpretation is consistent with the observation of protein resonances from lyophilized ROS membranes, as well as from hydrated purple membranes containing bacteriorhodopsin (16). For the latter two systems, there is no protein rotational diffusion with $1/\tau_R \approx 50$ kHz (17).

The $^1$C spin-lattice relaxation times, $T_1(C)$'s, of the multilamellar DLPC dispersions and the DLPC/rhodopsin recombinant membranes were found in general to be similar to those of the corresponding sonicated vesicles (not shown). The rotating-frame relaxation times, denoted by $T_{1p}(C)$, are summarized in Table 1 for the CH$_2$ resonances of the 50 wt% DLPC dispersions and the DLPC/rhodopsin recombinant membranes at a rotating field strength of $\gamma_C B_{1C}/2\pi = 30$ kHz. In general, the $T_{1p}(C)$ values were observed to be smaller than the corresponding $T_1(C)$ values by a factor of $\sim 10-20$, consistent with the frequency dependence of the $^2$H and $^1$C spin-lattice relaxation times proposed for lipid bilayers in the

<table>
<thead>
<tr>
<th>chain acyl segment</th>
<th>DLPC</th>
<th>DLPC/Rhodopsin (66:1)</th>
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<tbody>
<tr>
<td>C-2</td>
<td>32.4 ± 4.8</td>
<td>11.5 ± 2.2</td>
</tr>
<tr>
<td>C-3</td>
<td>28.6 ± 2.4</td>
<td>16.4 ± 4.9</td>
</tr>
<tr>
<td>C-4 → C-9</td>
<td>33.1 ± 2.5</td>
<td>14.7 ± 0.9</td>
</tr>
<tr>
<td>C-10</td>
<td>40.0 ± 3.7</td>
<td>17.1 ± 3.1</td>
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liquid crystalline phase (18,19). An example of the decay of the spin-locked $^{13}$C polarization in the presence of a 30-kHz rotating magnetic field is shown in Figure 2. Due to the instrumental limitations only the initial part of the decay, presumed to be of the form $S = S_0 e^{-t/T_{1p}(C)}$, was measured (20). Thus, it is not possible to ascertain at present whether a distribution of relaxation times exists. However, from $^2$H NMR studies, it is anticipated that the bulk of the unresolved $(CH_2)_n$ groups (corresponding to the C-4 to C-9 acyl chain segments) will behave similarly, and that any distribution of the $T_{1p}(C)$ times due to the orientational anisotropy of the relaxation (21,22) will be averaged in the 50 wt% H$_2$O multilamellar dispersions (23). Little variation in the $T_{1p}(C)$ values along the acyl chains is observed (cf. Table 1). No $T_{1p}(C)$ had a $B_{1C}$ dependence greater than square law.

The measured $T_{1p}^{1}(C)$ rates can, in general, include both spin-lattice and $^1$H-$^{13}$C spin-spin contributions, which must be distinguished before an analysis in terms of molecular dynamics is possible (20). The spin-spin contribution can be estimated from the magnitude of the local $^1$H fields in lipid bilayers. Taking a value for the residual $^1$H second moment of $M_2 \approx 4 \times 10^8$ rad s$^{-1}$ (24),

![Fig. 2: Decay of the bulk methylene (CH$_2$) magnetization of the multilamellar DLPC (•) and DLPC/rhodopsin (66:1) (●) dispersions as a function of time in a $^{13}$C spin-locking field of 30 kHz.](image)
we have $B_{10c} \approx (1/3M_2)^{1/2} \approx 1.8$ kHz, in agreement with the observation (vide supra) that the local $^1$H fields of membraneous lipids are sufficiently small to be averaged by spinning at 2.4 kHz in the absence of dipolar decoupling. By analogy to more extensive studies of crystalline and glassy polymers (8,20) where the local $^1$H fields are larger (3-10 kHz), we conclude that the $T_1$$^1$H(C) rates of lipid bilayers are largely spin-lattice in origin, and reflect the details of their molecular dynamics. These $T_1$$^1$H(C)'s can be sensitive to minor changes in either the motional rate or amplitude even though other motional parameters, characterizing dipolar or quadrupolar lineshapes, appear insensitive.

What are the slow motions providing the spin-lattice contribution to the measured $T_1$$^1$H(C) values of lipid bilayers? The observation that $T_1$$^1$P(C)$<T_1$(C) together with the measured frequency dependence of $T_1$(C) (19), suggests the presence of different motional components in lipid bilayers, each with characteristic amplitudes and correlation times (18). The low-frequency/small-amplitude components are expected to dominate the contribution to $T_1$$^1$H(C), whereas the high-frequency/large-amplitude components determine $T_1$P(C). In general, isotropic motions such as vesicle or liposome tumbling, or reorientation due to lateral diffusion can provide a lower limit to the frequencies of the lipid molecular or segmental motions that can be observed. For 50 wt% multilamellar dispersions, relatively slow relaxations are observed in the millisecond time regime (23); however, it can be safely concluded from the corresponding $^2$H and $^{31}$P NMR spectra (25) that such orientational fluctuations due to macroscopic transport do not yield significant spectral density above $\sim1$ kHz. Then, since the small amplitude lipid segmental fluctuations are believed to occur at frequencies greater than 170 kHz (26), it follows that the $^{13}$C $T_1$$^1$P(C) measurements are dominated by contributions from cooperative molecular or segmental fluctuations between 1 and 100 kHz. The top of this frequency regime, incidentally, can give rise to broad $^2$H NMR natural line-widths (short $T_2$ values). Thus, systems having short, easily measured $T_1$$^1$P(C)'s may be those containing components with difficult to detect $^2$H NMR echoes.
Regarding the influence of integral membrane proteins such as rhodopsin on the dynamics of lipid bilayers, we first note that the DLPC/rhodopsin recombinant membranes prepared by dialysis from dodecyltrimethylammonium bromide solutions form multilamellar structures similar in appearance to coarse lipid dispersions in the L_α phase (6). Thus, their physical properties can be compared directly. As can be seen (Fig. 2), the presence of rhodopsin in the recombinant DLPC membranes is associated with an increase in the lipid T_1ρ^1(C) rates measured at 30 kHz; i.e., the T_1ρ(C) times are shorter than those of the DLPC multilamellar dispersions. In general, the relaxation enhancement of the ^13C T_1ρ^1(C) rates due to rhodopsin appears greater than that of the corresponding T_1^1(C) rates (not shown; cf. Ref. 12), although the latter are also increased. Similar observations have been recently reported for other membrane systems (27). Since little change in the easily observed residual ^2H NMR quadrupolar splittings Δν_Q is seen for lipid bilayers in the presence of integral membrane proteins such as rhodopsin (28), the amplitudes of fluctuations above 170 kHz most likely remain unaltered. One possible explanation of all these results is that rhodopsin reduces the rates (but not amplitudes) of ultra high-frequency lipid motions thereby increasing spectral density in both the kHz and MHz regimes. Experiments designed to confirm this interpretation by measuring directly low-frequency amplitudes of motion in these systems are in progress.

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