Dynamics in a Protein-Lipid Complex: Nuclear Magnetic Resonance Measurements on the Headgroup of Cardiolipin when Bound to Cytochrome *c*

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ABSTRACT Deuterium and phosphorus nuclear magnetic resonance (NMR) has been used to investigate the dynamics of slow motional processes induced in bilayer cardiolipin upon binding with cytochrome *c*. ^{31}P NMR line shapes suggest that protein binding induces less restricted, isotropic-like motions in the lipid phosphates within the ms time scale of this measurement. However, these motions impart rapid transverse relaxation to methylene deuterons adjacent to the phosphate in the lipid headgroup and so did not feature strongly in the NMR line shapes recorded from these nuclei by using the quadrupolar echo. Nonetheless, motional characteristics of the headgroup deuterons were accessible to a dynamic NMR approach using the Carr-Purcell-Meiboom-Gill multiple-pulse experiment. Compared to the well-studied case of deuterons in fatty acyl chains of bilayer phosphatidylcholine, the motions determining the ^{2}H spin transverse relaxation in the headgroup of bilayer cardiolipin were much faster, having a lower limit in the 5–10 kHz range. On binding with cytochrome *c*, the T_2 effecting motions in the cardiolipin headgroup became faster still, with rates comparable to the residual quadrupolar coupling frequency of the headgroup deuterons (~25 kHz) and so coincided with the time scale for recording the quadrupolar echo (~40 μs). It is concluded that the headgroup of cardiolipin does not exclusively report localized dynamic information but is particularly sensitive to collective motions occurring throughout the bilayer molecules. Although the rates of collective modes of motion may be dependent on the lipid type in pure lipid bilayers, these low-frequency fluctuations appear to occupy a similar dynamic range in a variety of lipid-protein systems, including the natural membranes.

INTRODUCTION

Although a wide variety of motions are known to be available to lipid molecules in bilayer membranes (1), any direct role for these dynamic processes in membrane function remains unclear. Apart from their fast ($<10^{-7}$ s) intramolecular (2) and diffusive motions (3), the lipid molecules within the fluid bilayer can execute lower-frequency fluctuations in concert with its neighbors (1). These collective modes may involve large segments of the molecule fluctuating away from the bilayer normal, a process referred to as "order director fluctuations" (4, 5). The interest in these modes arises from their potential to transmit dynamic information through the membrane continuum and the fact that they occupy a time scale relevant to many biological processes (10^{-5}–10^{-3} s). Collective lipid fluctuations have consequently been believed to contribute to long-range protein-protein interactions within the membrane (6, 7) and, when culminating in large-amplitude, "out-of-plane" motions or undulations (8) in the bilayer surface, will have the ability to influence membrane-membrane interactions and the nature of cell-cell contacts (9, 10).

In recent work, we have used information obtained with solid-state nuclear magnetic resonance (NMR) techniques to describe the interactive behavior between cytochrome *c* and cardiolipin, the phospholipid localized within the mitochondrial inner membrane and the site of action of the protein. While significant perturbations in protein structure (11, 12) resulted in very diverse motional behavior at individual sites in this component (13), the binding appeared to induce an almost singular effect on motions in the lipid molecules, involving a strong enhancement of motions with rates in the tens of kHz range (13). While such rates are indicative of collective modes of motion, they were not prevalent at these frequencies in the pure lipid bilayers (13). A clearer distinction between the dynamics of collective motions in the lipid bilayers and protein-lipid complex was sought in the current study.

The slow motions induced in the cardiolipin by protein binding were previously detected from their contributions to NMR relaxation in the rotating-frame during a cross-polarization (CP) experiment (13). At typical field strengths used to irradiate the proton spins, this approach is particularly sensitive to motions in the tens of kHz range but has limitations for defining the distribution of these motional frequencies or even for specifying the dominant rates of motion. The current work attempts a more comprehensive analysis of these motional perturbations from NMR observation of the phosphates within the lipid headgroup, as well as of deuterons specifically introduced into this segment. The lipid headgroup is the primary site of attachment for the protein and should be the most sensitive to any localized perturbation induced by the binding. However, the headgroup glyceryl of

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Abbreviations used: NMR, nuclear magnetic resonance; CP, cross polarization; QE, quadrupolar echo; CPMG, Carr-Purcell-Meiboom-Gill; EFG, electric field gradient.

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cardiolipin, coupled at both ends to a phosphodiglyceride moiety, is relatively rigid compared with the monoesterified lipid headgroups (14) and so was expected to report equally well on motions involving extended segments of the molecule.

Combining observations of the static magnetic interactions (Zeeman) from both nuclei shows the ^2H NMR line shapes from the protein-lipid complex to be influenced by rapid transverse relaxation processes. However, an analysis of these dynamic NMR processes did yield an evaluation of the rates of slow motions induced by the protein.

MATERIALS AND METHODS

Protein-lipid complexes

Cytochrome *c* from horse heart (Type VI; Sigma Chemical Co.) was purified by ion-exchange chromatography on CM52 (Whatman) eluted with 65 mM phosphate buffer at pH 7.0 (15). The purified fraction was concentrated by ultrafiltration (YM5 membranes; Amicon) and dialyzed extensively against distilled water to remove phosphate. Aqueous protein concentration was determined by spectrophotometric analysis using a molar extinction coefficient of 2.95×10^4 at 550 nm for the reduced protein (16).

The deuterated cardiolipin analogue used, 1-[$^2H_2$]-1-(1,2-dilinoleoyl-*sn*-glycero-3-phosphoryl)-3-(1,2-dilinoleoyl-*sn*-glyceryl-3-phosphoryl)-*sn*-glycerol, is illustrated in Fig. 1 and was prepared by a general synthetic procedure developed for cardiolipins that will be described in detail elsewhere. Briefly, the headgroup was formed from glycerol in which deuterons were introduced specifically into the *sn*-1 position, by deutero-reduction of the appropriate methyl ester of the isopropylidene precursor. Coupling of the labeled glycerol to both diglyceride backbone segments was simultaneously achieved through phosphotriester formation, while protecting the *sn*-2 hydroxyl of the headgroup. The diglycerides had been prepared (17) with linoleoyl fatty acyl chains in both *sn*-1 and *sn*-2 positions, this being the fatty acid almost exclusively found in cardiolipin isolated from mammalian tissue (18). The final purified product showed less than 1 wt% impurity by thin-layer chromatography and exhibited optical activity equivalent to that of the natural lipid. The synthetic cardiolipin was obtained predominantly as the ammonium salt and was exchanged into the sodium form by washing an organic phase containing the lipid with aqueous solution containing a large excess of both NaCl and EDTA, the latter ensuring conversion of any divalent ion forms generated from exposure to column purification materials. The resulting solid was washed with double-distilled water and pelleted to remove reagents entrained from the salt exchange step and then finally lyophylized.

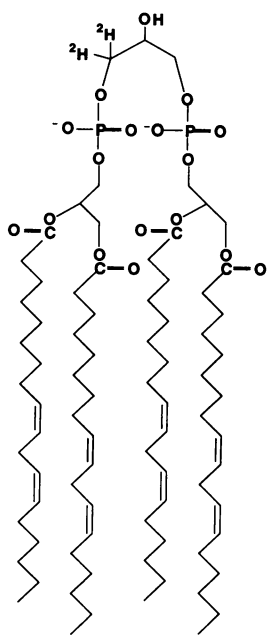

FIGURE 1 Chemical structure of cardiolipin synthesized with deuterons in the *sn*-1 position of the headgroup glycerol segment.

The hydrated samples of lipid or protein-lipid complex were prepared and maintained in an atmosphere of nitrogen to prevent oxidation of the polyunsaturated fatty acyl chains within the lipid. Batches of lyophylized cardiolipin (~150 mg) were hydrated with 0.3 ml of 20 mM cacodylate buffer at pH 6.0, containing 0.1 M NaCl and 5 mM EDTA. Hydrated cardiolipin was bound with increasing amounts of cytochrome *c*, added as a solution in the aqueous buffer (1 ml). After each addition, the protein-lipid mixture was allowed to equilibrate for at least 1 h with intermittent vigorous mixing and was then separated by ultracentrifugation (2×10^5 *g*) for 1 h. The resulting pellets were isolated for the NMR analysis, and the clear supernatants were removed for measurement of protein content. From these aqueous protein concentrations it was estimated that free protein represented, at most, 5% of the total in the pelleted complex and was often much less. Following NMR analysis, the components of the complex were unchanged according to thin-layer chromatography of the lipid and the ultraviolet/visible spectroscopic characteristics of the cytochrome *c*.

NMR methods

All NMR measurements were carried out on a Bruker MSL 400 spectrometer operating at 60.4 MHz for deuterium and 161.2 MHz for phosphorus. Probe heads were adapted to accommodate home-made solenoid coils that were used to enclose and irradiate the samples. Deuterium NMR transients were recorded using the quadrupolar echo (QE) pulse sequence (19), $(\pi/2)_x - \tau - (\pi/2)_y - \tau$ – acquire, employing $\pi/2$ pulse widths of 4–6 μs. Transverse ^2H-spin relaxation was measured with the same echo sequence by varying the interpulse delays (τ) and yielding a time constant for an exponential decay in echo intensity referred to as T_{2QE}. In addition, the pulse frequency dispersion in the transverse relaxation was analyzed with a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence adapted for the spin-1 nucleus (20, 21), $(\pi/2)_x - \tau - [(\pi/2)_y - 2\tau(\text{aquire})]_n$, where the pulse separation time, 2τ, is varied for each experiment, and the entire train of echos generated from the multiple pulsing was recorded throughout the sequence. A time constant for transverse relaxation under these conditions is designated T_{2CP}. The basic echo pulse sequence was also adapted for measuring spin-lattice relaxation times (T_1) by the inversion recovery technique, for which the recyle delay was at least 5 times T_1. Phosphorus NMR measurements were made using the Hahn echo with $\pi/2$ pulse widths of 5 μs and employing a 25-kHz field strength for proton decoupling. NMR transients were co-added with phase cycling suitable for minimizing artifacts in detection and were normally exponentially filtered with appropriate line broadening (30–100 Hz) prior to Fourier transformation.

RESULTS AND DISCUSSION

NMR line shapes

Spectral line shapes are first examined to evaluate motions faster than or comparable with the respective NMR time scales for motional averaging. The ^{31}P spectrum from the hydrated lipid alone (Fig. 2 *A, lower spectrum*) shows the high-field peak and low-field shoulder characteristic of a chemical shielding tensor which appears axially symmetric due to motions of the lipid in the bilayer configuration that are fast on the time scale for averaging of the phosphorus chemical shielding anisotropy (ms). This "powder" spectrum also contains a small shoulder around the isotropic shift of

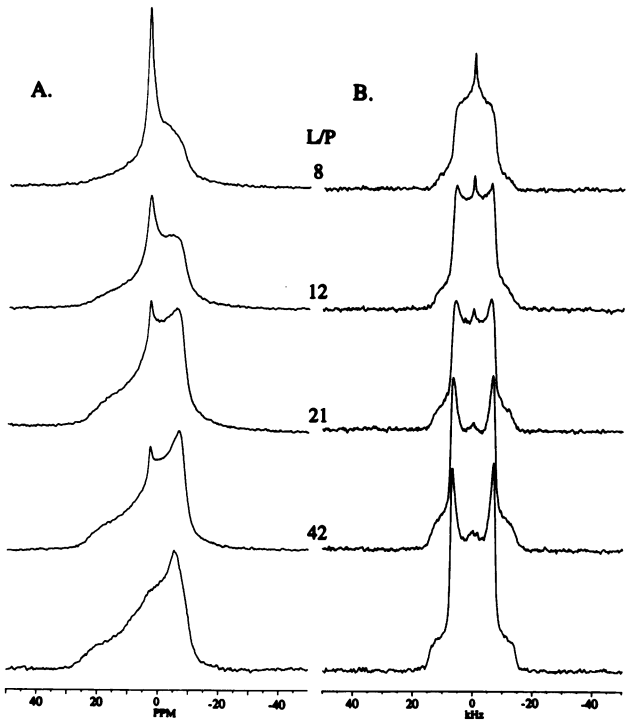

FIGURE 2 (*A*) Proton-decoupled ^{31}P NMR spectra from the hydrated cardiolipin alone (*lower*) and when complexed at the indicated molar stoichiometries with cytochrome *c* (*L/P*, lipid/protein molar ratio). All data obtained from 256 acquisitions, using a 1-s recycle time. (*B*) ^{2}H NMR spectra recorded from the headgroup deuterons in cardiolipin, from 10,000 acquisitions with a 50-ms recycle time. Spectrum from the hydrated lipid alone (*lower*) and for the protein-lipid complex at the indicated molar stoichiometries.

this nucleus (close to 0 ppm) where narrow components are frequently observed in the ^{31}P NMR spectra recorded from hydrated anionic phospholipids (22). As cytochrome *c* is incorporated into the complex, intensity in this central region of the spectrum develops into a pronounced spike, as shown in the series of spectra in Fig. 2 *A*, suggesting that a portion of the lipid phosphates are executing unrestrained, isotropic-like motions that are fast on the ms time scale of the ^{31}P NMR measurement.

The ^{2}H NMR spectrum from the hydrated lipid alone (Fig. 2 *B, lower spectrum*) shows the "Pake-doublet" pattern typically obtained from this spin-1 nucleus. The sharp peaks define the 90° orientations of the electric field gradient (EFG) tensor at the deuterons (also axially symmetric) with respect to the direction of the static magnetic field and provide a spectral splitting of around 13 kHz, similar to that reported previously for hydrated cardiolipin with deuterons introduced into the glyceryl methylenes by biosynthetic means (14). This splitting is reduced about tenfold compared with that for the static methylene bond, due to the fast rotations around the molecular long axis of the lipid molecule and, presumably, additional fast symmetric motions confined to the headgroup. Binding with increasing amounts of cytochrome *c* results in a progressive loss in definition of the 90° peaks, while increasing spectral intensity toward the center of the spectrum, as shown by the series of spectra in Fig. 2 *B*. This central intensity remains somewhat dispersed toward the edges of the spectra and is not strongly indicative of the introduction of isotropic motions, as implied by the ^{31}P NMR measurements. The developing line shape in fact appears more characteristic of two-site hopping motions between tetrahedral sites, which scale the residual coupling constant by one-half and cause the EFG tensor to appear fully asymmetric, as shown in a number of previous analyses (23–25). Line shapes similar to that recorded from the complex at the highest protein content (Fig. 2 *B, upper spectrum*) can be generated using exchange rates between the tetrahedral sites that are comparable to the residual coupling frequency of the deuterons, although motions in this "intermediate" regime frequently induce severe distortions in the spectral line shape, as shown in published examples (26). The recorded spectra show no distortions typical of restricted motions at these intermediate rates but instead reveal a progressive reduction in spectral intensity as bound protein is increased. These spectra therefore cannot be reliably used to interpret motions within the complex by comparison with conventional reference line shapes, which take no account of such losses in spectral intensity. The compliance with the line shapes discussed above is also highly suspect since any true departure from axial symmetry in EFG tensor would require a restricted reorientation in the major axis of motional averaging (director, or molecular long axis), a situation that is difficult to envisage within these "fluid" lipid systems.

It is unlikely that the motions observed on the time scale of the ^{31}P NMR measurements were simply too slow to be detected by ^{2}H NMR, since the effective motional averaging of the EFG tensor for the headgroup deuterons and the relatively high field used to observe the ^{31}P shielding anisotropy mean that only a very narrow frequency window exists (around 10–20 kHz) where motions can convert from "fast" to "slow" regimes between the two experiments. It is more likely that motions will fall into the "intermediate" regime on the ^{2}H NMR time scale, and direct evidence of this is obtained from the relaxation analyses presented below.

^{2}H-spin relaxation

The spectral intensity recorded from the headgroup deuterons decayed by about one-half over the range of protein to lipid concentrations used as illustrated in Fig. 3 *A*. This was not due to any change in the equilibrium magnetization of lipid deuterons since their spin-lattice relaxation was not significantly altered upon complexation with the protein, as shown by the T_1 data also presented in Fig. 3 *A*. The intensity changes are closely paralleled, however, by effects of the protein on the transverse relaxation rate of the ^{2}H spins, which shows a dramatic enhancement on binding with the protein, as indicated by the T_{2QE} data in Fig. 3 *B*. Transverse relaxation rates from separate samples of the hydrated lipid showed reasonable reproducibilty (<15%), although greater variability (up to 30%) was generally observed between measurements on separate preparations of the complex. This vari-

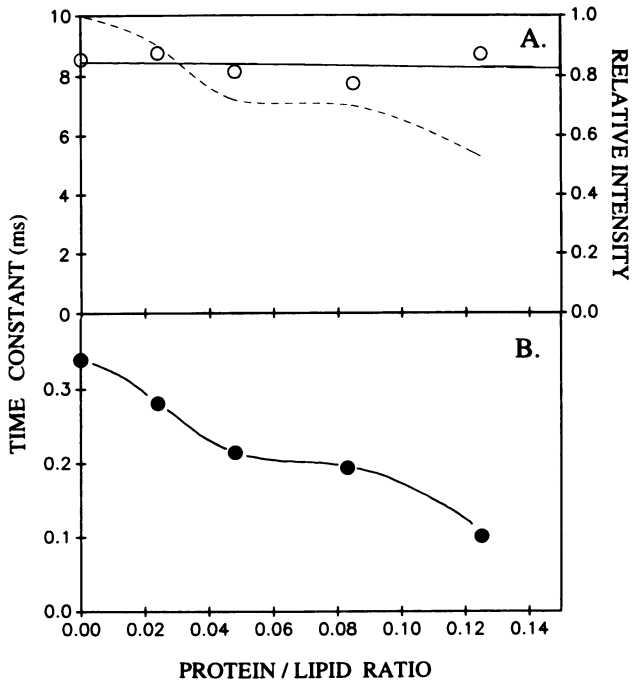

FIGURE 3 ^{3}H NMR characteristics of the headgroup deuterons in cardiolipin as a function of the molar ratio of protein to lipid in the complex. (*A*) Normalized spectral intensity (*broken line, right ordinate*) relative to that recorded in the absence of protein and spin lattice relaxation times (T_1; ○, solid line). (*B*) Transverse relaxation times recorded in the QE experiment (T_{2QE}) measured for the spectra in Fig. 2 *B*.

ability, which may be due to some differences in the extent of dehydration of the cardiolipin bilayers caused by binding with the protein, does not interfere with the overall trends described here for the data or invalidate any of the arguments presented.

Since we detect no significant effect on fast (~ns) motions affecting T_1, there would not appear to be any overall restriction in lipid motions that increase the low and zero frequency contributions to the transverse relaxation. Rather, efficient transverse relaxation is predicted to arise from the introduction of frequencies specifically within the intermediate regime and occurring within the time scale of the echo experiment (~40 μs). To unequivocably demonstrate the enhancement of motions within this frequency range, transverse relaxation was analyzed as a function of pulse frequency in the CPMG experiment. Here, a comparison is made of relaxation rates measured from the QE ($1/T_{2QE}$) and CPMG ($1/T_{2CP}$) methods, as presented previously for the spin-1 quadrupolar nucleus (20) and summarized here. In the limit where molecular motion is fast with respect to the NMR time scale of molecular motional averaging (10^{-4}–10^{-3} s), the time constant for the exponential transverse relaxation measured conventionally with the QE is inversely proportional to the correlation time, τ_c, for a single molecular motion dominating T_2 and can be expressed as (27)

$$\frac{1}{T_{2QE}} = \Delta M_2 \tau_c \qquad (1)$$

where ΔM_2 is the reduction in the second moment of the spectrum due to the motion.

A general expression governing transverse relaxation of quadrupolar nuclei under the influence of a train of refocusing pulses, as in the CPMG experiment, has been developed by Blicharski (28) and is as follows:

$$\frac{1}{T_{2CP}} = \Delta M_2 \tau_c \left[1 - \frac{\tau_c}{\tau} \tanh\left(\frac{\tau}{\tau_c} \right) \right] \qquad (2)$$

where τ is the interval between refocusing pulses. At the short correlation time limit, where $\Delta M_2 \tau_c^2 << 1$ or, alternatively, $\tau_c << \tau$, the time constant attains the minimum value, $T_{2CP} \approx T_{2QE}$. At the opposite time limit, where $\Delta M_2 \tau_c^2 >> 1$ or, in experimental terms, the pulse separation time is now rapid compared with the molecular motion ($\tau_c >> \tau$), then random phase does not accumulate as rapidly and the now lengthened time constant can be expressed as (20)

$$\frac{1}{T_{2CP}} \approx \frac{\Delta M_2 \tau^2}{3\tau_c} + \frac{1}{T_2'} \qquad (3)$$

where $1/T_2'$ accounts for contributions to the relaxation rate from fast processes. Thus a comparison of transverse relaxation within the QE and CPMG experiments allows the limit of τ_c to be defined in terms of the pulse separation time, referred to subsequently as 2τ, which can be varied in the CPMG experiment in an attempt to define explicitly the frequencies of motion that dominate transverse relaxation.

Previous reports of this analysis for phospholipid bilayers have been confined to deuterons located within saturated fatty acyl chains of phosphatidylcholine (20, 29). Data were also obtained from this sample type for the current study and are shown in Fig. 4 *A* to illustrate this "typical" response and assist in discussing behavior of the experimental system incorporating cardiolipin. Transverse relaxation from the QE experiment could be represented as a single exponential decay function, as shown from the data in Fig. 4 *A* (QE). Exponential transverse relaxation in the QE experiment is generally a reasonable approximation for deuterons in bilayer lipid, although this is not strictly justified on theoretical grounds (20, 27, 30). However, the relaxation data from the CPMG experiment show marked nonexponential behavior. As noted previously for measurements on samples similarly comprising a random orientation of bilayers (20), the relaxation rates exhibit a strong dependence on 2τ only at shorter times and become independent of 2τ after a few hundred microseconds of accumulated relaxation time. However, CPMG relaxation rates appear to remain exponential for acyl chain deuterons within macroscopically oriented bilayers (29), although this also has no theoretical basis for long correlation times (reviewer's note and Eq. 3). By deducing relaxation rates from the initial slopes in the plots, as done previously (20), it is clear that the rates remain appreciably slower than the rate recorded in the QE experiment. The correlation times for molecular motions determining transverse relaxation in this case are therefore concluded to be far

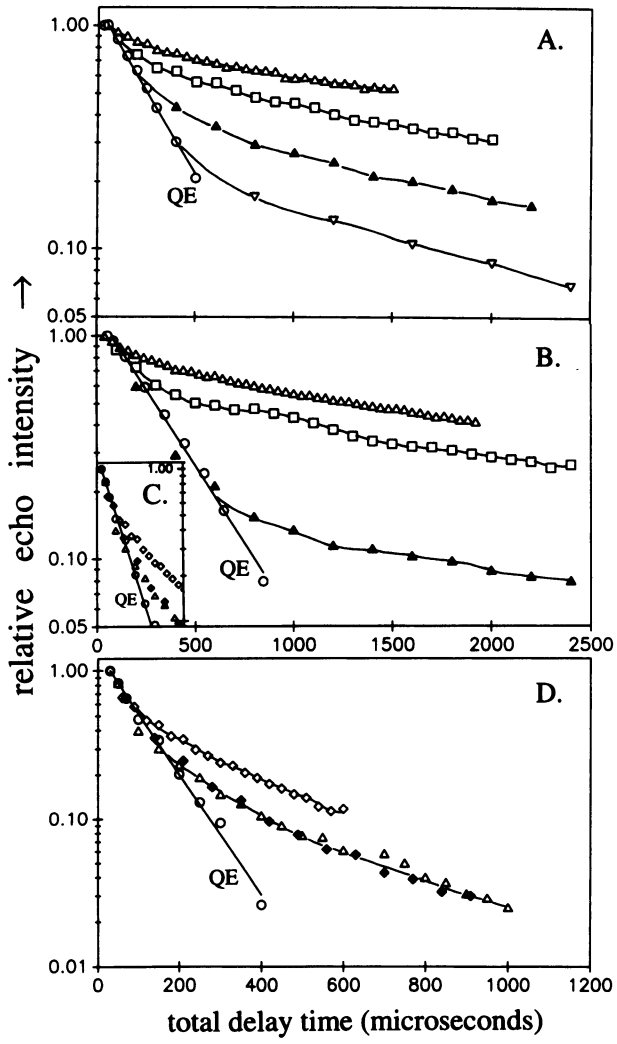

FIGURE 4 Intensity of NMR echoes, relative to that recorded at the shortest delay time, as a function of total relaxation delay in the QE (*QE*; ○, linear plots) and the CPMG experiment (data on nonlinear plots) for deuterons in (*A*) bilayers of 1-stearoyl-2-[9-$^2H_2$]stearoyl-*sn*-glycerol-3-phosphorylcholine; (*B*) the headgroup of cardiolipin in bilayers alone; (*inset C*) initial relaxation data from the cardiolipin bound at a 12:1 molar ratio with cytochrome *c*; (*D*) the full range of data recorded from this protein-lipid complex on an expanded time scale. Measurement made at 55°C for the saturated lipid (*A*) or at 25°C for the samples containing the deuterated cardiolipin. Pulse separation times (2τ) for CPMG data in (*A*) 50 μs (△), 100 μs (□), 200 μs (▲), 400 μs (▽); (*B*) 40 μs (△), 100 μs (□), 200 μs (▲); (*D*) 30 μs (◇), 50 μs (△), 70 μs (◆). The QE data give T_{2QE} values of 312 μs from (*A*), 339 μs from (*B*), and 101 μs from (*C*).

in excess of 400 μs and possibly on the order of milliseconds, as proposed by others for this lipid type (29).

Deuterons within the headgroup of cardiolipin exhibit a rate of transverse relaxation in the QE experiment similar to that of the chain deuterons studied here, but the relaxation process reveals a very different pulse frequency dependence in the CPMG analysis. The initial slopes described by the CPMG data in Fig. 4 *B* begin to approach that from the QE relaxation data at the shorter pulse separation times and become superimposable at $2\tau = 200$ μs. This is the first reported instance where it has been possible to satisfy the condition $T_{2CP} \approx T_{2QE}$ for deuterons within the hydrated lipid bilayers and allows the dynamics of motions affecting T_2 relaxation in this case to be specified with greater certainty. The observations indicate that these motional components in bilayers of cardiolipin alone have a high density and an effective lower limit within the 5–10-kHz range.

The faster ^{2}H-spin transverse relaxation rates observed on binding of the protein are illustrated by the data shown in the inset (*C*) to Fig. 4, and these are presented on an expanded time scale in Fig. 4 *D*. Now the initial rates of the CPMG relaxation tend toward the rate of QE relaxation at very short pulse separation times and become limiting in the range of 30–50 μs, since no further increase in the rate was observed with longer pulse spacings. The onset of 2τ-independent relaxation also occurs at shorter times for the protein-lipid complex, resulting in a rather short period where relaxation rates coincide between the two experiments. It is clear, however, that rates for motions dominating transverse relaxation in the protein-lipid complex now lie in the intermediate regime with respect to the residual quadrupolar coupling frequency (~25 kHz) and will interfere critically with acquiring the QE. All the above trends observed in the CPMG analyses were well reproduced for duplicated samples of lipid and protein-lipid complex.

The principal motions effecting ^{2}H-spin transverse relaxation in the protein complexed lipid have a lower limit in the 20–30 kHz range and are considered less restrained than indicated by the observed ^{2}H NMR line shapes. Of the variety of motions executed by the lipid headgroup in the protein-lipid complex, those expressing higher degrees of freedom (i.e., more isotropic) are evidently being preferentially dephased and so suffer drastic losses during recording of the QE. This consequence of less restricted, isotropic-like motions within the intermediate regime was predicted in previous discussions of ^{2}H NMR line shapes (24) but has yet to be demonstrated in spectral simulations.

The slow motions revealed in the lipid bilayers by the CPMG experiment were initially attributed to whole molecule diffusion around bilayer surfaces (20) and so would be highly sensitive to the morphological features of the lipid dispersions. However, the subsequent studies on oriented bilayers (29) argue against this diffusive model and instead emphasize the importance of long-range collective fluctuations in the bilayer surface, which are a consequence of the order director fluctuations. Some consideration must be given here to the morphology of lipid dispersions studied here, particularly in view of the unrestrained motions induced in this component by protein binding. De Kruijff and Cullis (31) reported that cytochrome *c* can induce the formation of nonbilayer states in hydrated dispersions of cardiolipin. The changes in the ^{31}P spectra reported here are not indicative of a conversion to the hexagonal H_{II} phase, as observed by these workers (31). Diffusive motion around the more highly curved interfaces associated with the classical H_{II} phase have also been shown to be rapid on the ^{2}H NMR

time scale (32) and so are faster than the reorientational motions considered here. De Kruijff and Cullis (31) also describe intramembrane particles within the complex, perhaps representing structures that are intermediate between the lamellar and H_{II} states. These structures would also be sufficiently small to allow rapid whole molecule reorientation on the ^2H NMR time scale. Diffusive contributions on a relevent time scale would therefore need to involve changes in long-range morphology such as the overall size of the liposomal dispersion, rather than some shorter-range reorganization into nonbilayer states. Previous microscopic observation of similar complexes (31, 33) does not appear to indicate any decrease in the overall size of the lipid dispersions from protein binding that could contribute to the effects on NMR line shapes and relaxation observed here. Indeed, judging by the physical behavior of the complex during the preparative procedures, protein binding induces more highly fused supramolecular assemblies of lipid that are much more difficult to disperse than discrete liposomal structures. Since there is no evidence for morphological changes that account for the current observations, some alternative interpretion can be sought in terms of the collective modes of motion within bilayer membranes.

The rates of motion affecting T_2 within the headgroup of cardiolipin alone in the lipid bilayers were at least an order of magnitude faster than has been predicted for the slow motions within saturated fatty acyl chains of phosphatidylcholine in the liquid-crystalline bilayer (29). Nonetheless, the observed rates of motion remain within the low-frequency realm that characterize the collective modes. Watnick and co-workers (7) analyzed the transverse relaxation of deuterons at several locations within dimyristoylphosphatidylcholine and concluded that collective fluctuations were largely confined to the hydrophobic interior of the bilayer, with the outermost lipid segments providing an "anchor" from which these motions can propagate. It is not assumed from this model that the collective fluctuations detected within cardiolipin must be confined to the headgroup region. It is more likely that the rigidity of the headgroup in cardiolipin enables its motions to become strongly coupled to those within the chain segments. Also, weak cohesive forces between the divalent headgroups of cardiolipin, which have a small cross-sectional area compared with the hydrophobic chain segments in this lipid, would assist in the slower headgroup motions being dominated. These factors can also contribute to the headgroup becoming substantially disordered under the influence of high-density collective fluctuations, as in the protein-lipid complex.

The above conclusion that the headgroup of cardiolipin may be especially sensitive to the low-frequency collective motions within the bilayer is supported by closely related studies (34) which showed that the binding of cytochrome *c* to phosphatidylserine in bilayers enhanced the transverse relaxation of deuterons introduced into the fatty acyl chains of the lipid, but not for deuterons within the headgroup segment of this monoesterified lipid. In contrast to the current observations of the cardiolipin headgroup, the monoesterified headgroup of phosphatidylserine could not be considered to partake in collective fluctuations that are induced or modulated by protein binding, even when binding is confined to the surface regions of the bilayer. The motional processes detected from the cardiolipin headgroup in the protein-lipid complex are consequently expected to reflect collective modes that are active throughout the lipid molecules. The emphasis placed here on rates in the tens of kHz range for motions throughout the complexed lipid is consistent with our less quantitative but more extensive analysis based on NMR relaxation in the rotating frame (13). However, unlike the dynamics described above for the pure lipid bilayers, the frequency range highlighted by the current analysis can be considered quite typical for collective modes in protein-lipid systems. Judging by circumstantial or more direct evidence provided by NMR relaxation behavior, collective fluctuations in the tens of kHz range are a common feature of many bilayer systems incorporating peptides, including gramicidin (35) and melittin (36), or proteins, of which natural membranes are the best example (37). Rather than reporting on any specific effects from binding of an "extrinsic" membrane protein, the current work lends support to a proposal made previously (34), that the major impact of proteins on bilayer dynamics is their nonspecific influence on the nature of collective motions (order director fluctuations). It is suggested here that the rates of these collective motions are increased compared with those modes in pure lipid bilayers to occupy a similar frequency range in many diverse peptide or protein-lipid systems. Such a general effect on bilayer dynamics could be interpreted simply as a consequence of the lipid packing being interrupted to reduce effectively the cooperative length of slow motions in the bilayer.

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