Acidic Phospholipid Bicelles: A Versatile Model Membrane System

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ABSTRACT With the aim of establishing acidic bicellar solutions as a useful membrane model system, we have used deuterium NMR spectroscopy to investigate the properties of dimyristoyl/dihexanoylphosphatidylcholine (DMPC/DHPC) bicelles containing 25% (w/w in H_2O) of either dimyristoylphosphatidylserine (DMPS) or dimyristoylphosphatidylglycerol (DMPG). The addition of the acidic lipid component to this lyotropic liquid crystalline system reduces its range of stability because of poor miscibility of the two dimyristoylated phospholipids. Compared to the neutral bicelles, which are stable at pH 4 to pH 7, acidic bicelles are stable only from pH 5.5 to pH 7. Solid-state deuterium NMR analysis of d_{54}-DMPC showed similar ordering in neutral and acidic bicelles. Fully deuterated DMPS or DMPG is ordered in a way similar to that of DMPC. Study of the binding of the myristoylated N-terminal 14-residue peptide μ-GSSKSKPKDPSQRR from pp60$^{v\text{-}src}$ to both neutral and acidic bicelles shows the utility of these novel membrane mimetics.

INTRODUCTION

Discoidal mixed micelles composed of a bilayered phospholipid segment surrounded by a rim of surfactant (Small, 1967) were originally proposed by Prestegard and co-workers (Ram and Prestegard, 1988) as a membrane model system for use in structural NMR studies. The most commonly used are bicelles (Sanders and Landis, 1995). Bicelles contain zwitterionic dimyristoylphosphatidylcholine (DMPC), which forms a small circular bilayer, and dihexanoylphosphatidylcholine (DHPC), which coats the rim of the bilayer and isolates the hydrophobic core from water. The size of the bicelles increases as the molar ratio $q = n_l/n_s$ between the long-chain and short-chain phospholipid increases (Carey and Small, 1970; Vold and Prosser, 1996), and for solutions containing 2–40% (w/w) lipid at $2 < q < 10$, a discotic nematic liquid crystalline phase is formed. In magnetic fields of the strength typically employed in NMR spectroscopy (1–20 T), the bicelles align in a collective fashion with their normal perpendicular to the field (Sanders and Prestegard, 1990). Bicellar solutions may consequently be used as a macro ordered matrix that permits residual dipolar and quadrupolar splittings to be used in NMR structure determination (Emsley and Lindon, 1975) of solutes. The use of lyotropic liquid crystals was proposed for structural work in biology more than 30 years ago (Lawson and Flautt, 1967; Forrest and Reeves, 1981), but phospholipid bicelles have proved especially valuable for this purpose, because enzymes have been shown to maintain activity in this environment (Sanders and Landis, 1995).

At relatively high lipid concentration (10–20% by weight), bicelles with $q = 3–4$ and diameter $\approx 400–600$ Å (Vold and Prosser, 1996) have been used to examine the structure and the orientation of membrane-associated peptides and proteins (Sanders and Prestegard, 1990; Sanders and Landis, 1995; Sanders et al., 1994; Howard and Opella, 1996; Losonczi and Prestegard, 1998; Struppe et al., 1998). Smaller bicelles with $q = 0.5$, which form an isotropic solution, have proved suitable for high-resolution structural studies of membrane-bound peptides (Vold et al., 1997). Finally, bicellar solutions with lower lipid concentration (2–5%) have been proposed (Tjandra and Bax, 1997) as a medium for inducing weak, macroscopic ordering of non-membrane-interacting proteins, because the induced dipolar couplings may be used as additional constraints in structure determination.

With one exception (Struppe et al., 1998), only charge neutral bicelles composed of phosphatidylcholines have been used for NMR studies of membrane-bound polypeptides. However, many biologically relevant membranes contain negatively charged lipids, also called acidic phospholipids. Thus it seemed important to demonstrate the preparation and utility of bicelles containing acidic phospholipids. In this paper we describe the experimental protocol required for successful incorporation of acidic phospholipids into DMPC/DHPC bicelles. We chose to experiment with phosphatidylglycerol (PG) and phosphatidylserine (PS) because these lipids are the most abundant acidic phospholipids in, respectively, prokaryotic and eukaryotic membranes (Voet and Voet, 1997). In addition, their headgroup sizes are similar to that of phosphatidylcholine (PC), and accordingly the main phase transition temperatures T_t from the lamellar gel phase L_β or ripple phase $P_{\beta'}$ to the fluid lamellar phase L_α are similar. T_t depends strongly on repulsive interactions in the hydrophobic part of the bilayer and consequently on the size and structure of the acyl chains. But T_t is also affected by the size, polarity, and ionization state of the headgroup and the resulting specific intermolecular electrostatic interactions and hydrogen bonds (Boggs, 1984; Cevc and Marsh, 1987).

T_t for DMPG (24°) matches that of DMPC (25°) for fully hydrated bilayers at pH > 2.9, the pK_a of the PG phosphate

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group (Träuble, 1976). Thus bicelles containing mixtures of DMPC and DMPG should be relatively simple to prepare and the resulting nematic phases reasonably stable. On the other hand, the preparation of bicelles containing a mixture of DMPC and DMPS is likely to be less straightforward for two reasons: 1) T_t for DMPS is 13 K higher than that of DMPC at neutral pH, which suggests miscibility problems (Marsh, 1990); and 2) the $pK_a = 5.5$ for the PS carboxyl group is close to the pH range where many NMR experiments would usually be performed to avoid rapid exchange of nonprotected amide protons.

In this paper we make some observations on the preparation and handling of DMPC/DHPC bicellar solutions containing 25 mol% DMPX (X = S or G). We describe the properties of the bicellar solutions as a function of time, temperature, pH, and ionic strength and report briefly on the effects of Ca^{2+} and Yb^{3+} ions. Finally we illustrate the utility of the acidic bicelles as membrane mimetics for structural work in a study of the insertion and ordering of the N-terminal myristoyl group of the positively charged 14-residue peptide μ-GSSKSKPKDPSQRR from the tyrosine kinase $pp60^{v\text{-}src}$.

MATERIALS AND METHODS

Materials

Lipid samples

1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-d_{54}-*sn*-glycero-3-phosphocholine (DMPC-d_{54}), 1-myristoyl-d_{27}-2-myrsitoyl-*sn*-glycero-3-phosphocholine (DMPC-d_{27}), 1,2-dimyristoyl-*sn*-glycero-3- [phospho-*rac*-(1-glycerol)] (DMPG), 1,2-dimyristoyl-d_{54}-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG-d_{54}), 1,2-dimyristoyl-*sn*-glycero-3-[phospho-L-serine] (DMPS), and 1,2-dimyristoyl-d_{54}-*sn*-glycero-3-[phospho-L-serine] (DMPS-d_{54}) were all purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Deuterium-depleted water and deuterium oxide were obtained from Cambridge Isotope Laboratories (Cambridge, MA).

Peptide

The 14-residue peptide myr-d_{27}-GSSKSKPKDPSQRR was synthesized using fluoren-9-ylmethoxy carbonyl chemistry on a PerSeptive Biosystems solid-state peptide synthesizer, following a procedure described elsewhere (Meininger et al., 1995). The free carboxyl group of myristic-d_{27} acid (CDN) was coupled to the amino terminus by a standard peptide synthetic scheme using HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) as the activator in the coupling reaction. The peptide was purified by high-performance liquid chromatography (Waters) on a C18 column with a 0.1% trifluoroacetic acid (TFA)-acetonitrile gradient, and its identity and purity were confirmed by electrospray mass spectrometry.

Sample preparation

Bicellar samples with $q = \{[DMPC] + r\,[DMPX]\}/\{[DHPC](1 + r)\}$ with X = S or G, r = [DMPX]/[DMPC], in the range q = 3.2–3.9, were prepared by weight. As described previously (Struppe et al., 1998), a homogeneous slurry containing 20% (w/w) of the dimyristoylated phospholipids in deuterium-depleted water can normally be prepared by vortexing, centrifugation, and cycling the temperature between ~45–65°C and ~10°C. If this is not sufficient, freeze-thaw cycles combined with centrifugation and/or sonication may be used to break up visible, gel-like aggregates. Sonication with a small tip sonicator (Fisher Dismembrator 650) at the lowest possible power level is also a useful procedure for preparation of a homogeneous slurry without any apparent differences observable in the final bicellar samples.

All phospholipids are hygroscopic and pick up water if they are not handled properly under inert gas atmosphere. Based on earlier studies on lipid hydration (Jendrasiak and Hasty, 1974) and dehydration (Chapman et al., 1967), the water content of DMPC at 80% relative humidity is estimated to be 5 mol H_2O/mol phospholipid under normal laboratory conditions. Under dry conditions two H_2O molecules remain per DMPC molecule (Hauser et al., 1981). Given these difficulties, it is strongly recommended that ^{31}P NMR spectra be used to determine the actual value of q if necessary (Sanders and Schwonek, 1992; Tjandra and Bax, 1997). DHPC picks up water extremely fast when exposed to the laboratory atmosphere (~70% rel. humidity) and becomes visibly glassy within the first minute. A possible systematic error of the determined weight from uncontrolled hydration was eliminated by using a glove bag under an inert gas atmosphere to transfer the dry DHPC to a sample container with known weight. Once the weight of the DHPC is known, the sample container can be opened and the water added (by weight) at normal laboratory atmosphere. This procedure yields bicellar samples with highly reproducible properties. On the basis of numerous sample preparations, the best mixing of the components was achieved by first vortexing the highly viscous bicelle solution at a higher temperature (25–30°C) followed by vortexing at a lower temperature (below 20°C), to generate the low-viscosity isotropic phase. The ionic strength was adjusted by adding aliquots of concentrated salt solutions. The chosen pH was obtained by titration with 1 N NaOH or HCl solutions. All samples were stored at −20°C when not in use.

Samples of pp60 src peptide in bicelles were prepared by mixing solid peptide with dry long-chain phospholipid before preparation of bicelle solutions. The peptide-to-total lipid molar ratios were 1:40. After a uniform suspension was obtained, the DHPC solution was added and the final pH was adjusted to 5.5 by the addition of 1 M NaOH.

Flat-bottomed NMR tubes (25 mm long, 5 mm o.d.; Wilmad) were filled with 220 μl of bicelle solution and sealed with rubber plugs. Well-aligned bicellar NMR spectra were observed within a few minutes after insertion of the sample in the magnetic field at temperatures between 35°C and 40°C. Typically, the NMR samples were equilibrated for 30 min before the start of an experiment. Samples with $q \geq 3.5$ were most readily aligned when kept at 41°C for at least 15 min in the magnetic field. After temperature changes, the samples were again equilibrated for 30 min before data acquisition.

Thin-layer chromatography (TLC) was used to check for hydrolysis products. The water from the bicelle samples was removed by speed vac, and the remaining lipid was resuspended in 70:30 methanol:chloroform before spotting on the plates. We used 200-μm silica gel 60 analytical TLC plates (Selecto Scientific) with a 65:25:4 chloroform:methanol:ammonium hydroxide solvent system. Lipid spots were identified using a standard phosphorous stain (Kates, 1972).

NMR experiments

Deuterium quadrupole echo spectra were obtained either on a General Electrics GN500/Tecmag or on a Chemagnetics 250/360 spectrometer equipped with a 5.9-T or 8.6-T Oxford Instruments magnet, respectively. Both spectrometers utilize ENI LPI-10 rf amplifiers connected to home-built solid-state probes tuned to 38.4 MHz or 55.3 MHz, respectively. Between 1024 and 8192 transients were accumulated, using the standard quadrupolar echo sequence $\tau/2$-τ_1-$\tau/2$-τ_2-*acq* (Davis et al., 1976), where $\tau_1 = 50\ \tau$s and $\tau_2 = 35\ \tau$s, with a 1-s repetition time and $\tau/2$ pulse widths

of 6.5 μs or 2.1 μs at 38.4 and 55.3 MHz, respectively. A LakeShore 91C controller provided a sample temperature that was stable within $\pm$0.1°C, and the temperature gradient across the sample was less than 0.1°C. The transients were acquired with a high sampling rate of 500 kHz to facilitate identification of the quadrupole echo maximum. Data points (4kB) were collected for each free induction decay (FID). The accumulated signal was corrected for DC offset, fractionally left shifted, zero-filled, and multiplied by a 100-Hz exponential apodization function before Fourier transformation.

RESULTS AND DISCUSSION

Preparation and stability of acidic bicelles

Attempts to prepare acidic bicelles without any DMPC, e.g., DMPG/DHPC or DMPS/DHPC bicelles with $q = 3$, were not successful. Compared with DMPC/DHPC bicellar solutions, which have a low viscosity phase below 25°C at pH 5–6, both DMPS/DHPC and DMPG/DHPC mixtures stay highly viscous below room temperature. They are opaque rather than transparent, and they do not align in the magnetic field between 30°C and 50°C. Unsuccessful attempts were also made to mix the separately prepared DMPG/DHPC and DMPS/DHPC solutions with DMPC/DHPC solutions by vortexing and/or sonication. Acidic bicelles were successfully prepared by first mixing (vortexing) the two long-chain phospholipids with water at appropriately high temperatures (~40°C) to produce a homogenous slurry and then adding the DHPC solution, as described in the experimental section.

Deuterium quadrupole echo spectra of $q = 3.2$ DMPC/DMPS/DHPC bicellar solutions with 20% lipid and $r = 0.33$ were recorded as a function of time in the magnetic field ($B_0 = 5.9$ T); these are shown in Fig. 1. In column A are presented spectra of a pH 4.7 sample containing DMPC-d_{54}, and columns B and C show spectra of two samples containing DMPS-d_{54} recorded at pH 4.7 and pH 5.5, respectively. At pH 4.7, the plateau splitting, which can be measured as the separation between the outermost lines in the spectrum, and which arises from the C_2-C_5 methylene groups of DMPC, increases slightly over time. A small loss of resolution was also observed during the first few hours. After 3 days no further changes were observed. The spectra in column B were collected on a formally identical sample, except that the DMPS instead of the DMPC was chain-perdeuterated. Comparison of columns A and B shows that the DMPS is undergoing dramatic changes in less than 24 h. Loss of resolution after 24 h was followed by the unmistakable appearance of a deuterium powder pattern as well as an isotropic central resonance. This indicated a loss of ordering of the DMPS component of the bicelles that was observed when the DMPS was chain perdeuterated. Both samples A and B were observed to undergo phase separation, and upon removal from the magnetic field, the samples were indeed observed to be opaque rather than clear. The two phases were separated by centrifugation and could not be remixed by vortexing, freeze-thaw cycling, or sonicating. TLC of the pellet demonstrated the formation of hydrolysis products (lysophospholipids). A loss of ordering of only the DMPS-d_{54} signal and not the DMPC-d_{54} signal was observed in the NMR experiments. These two observations are most consistent with the idea that the lysophospholipids are due to hydrolysis of the DMPS specifically. Fortunately, the DMPS-containing bicelle solutions were stable for more than 2 weeks at temperatures between 35°C and 40°C if the pH was kept at 5.5, where no phase transition or lipid hydrolysis was observed (Fig. 1 *C*).

Similar experiments were also performed for bicelles with DMPG instead of DMPS, and, surprisingly, these bicelles also showed the formation of lysophospholipids over time and loss of order in the DMPG-d_{54} signal but not the DMPC-d_{54} signal. Loss of stability below pH 5.5 was due to increased hydrolysis of the anionic phospholipid component (data not shown). Subsequent experiments with both DMPS- and DMPG-containing bicelles showed that at pH 5.5, the ionic strength could be varied by up to 1 M monovalent ions without loss of stability (data not shown).

Characterization of acidic bicelles

Fig. 2 shows the q-dependence of the quadrupolar splitting, Δ, at three temperatures, 34.2°C (*triangles*), 36.2°C (*squares*), and 39°C (*diamonds*), for three different sites along the perdeuterated myristoyl chains of DMPC-d_{54} for neutral bicelles (*open symbols*) and acidic bicelles (*solid symbols*) with 25% DMPG or DMPS, all at pH 5.5. Within the estimated uncertainties in q ($\pm$5%) and Δ (1–5%), no significant difference was observed between the splittings in acidic and neutral bicelles. In agreement with previous studies of neutral bicelles (Sanders and Schwonek, 1992), the quadrupolar splittings observed in the acidic bicelles increased for $q < 3.5$ as the bicelles grow in size, but above $q = 4$ the splittings were essentially independent of q, indicating no further increase of the discoidal ordering of the bicelles. If one calculates the diameter of the bicelles according to that predicted for ideality (Vold and Prosser, 1996), the data in Fig. 2 show that bicellar order levels off above $d = 25$ nm. This is the result that was obtained for neutral bicelles from both order parameters. The predicted diameters have recently been supported by electron microscopy images (Yu et al., 1999). The limiting value, $\Delta = 22$ kHz, for the plateau is slightly less than that observed for DMPC bilayers aligned on glass plates (Opella and Morden, 1989) and for multilamellar DMPC vesicles (Struppe, unpublished results). It is increasingly difficult to reach orientational alignment equilibrium for bicellar samples in the magnetic field as the bicellar size increases with q, because alignment proceeds more and more slowly. The scatter in Δ observed in Fig. 2 for individual samples above $q = 4$ supports this hypothesis. Some of the samples were examined several times over a period of more than 6 months,

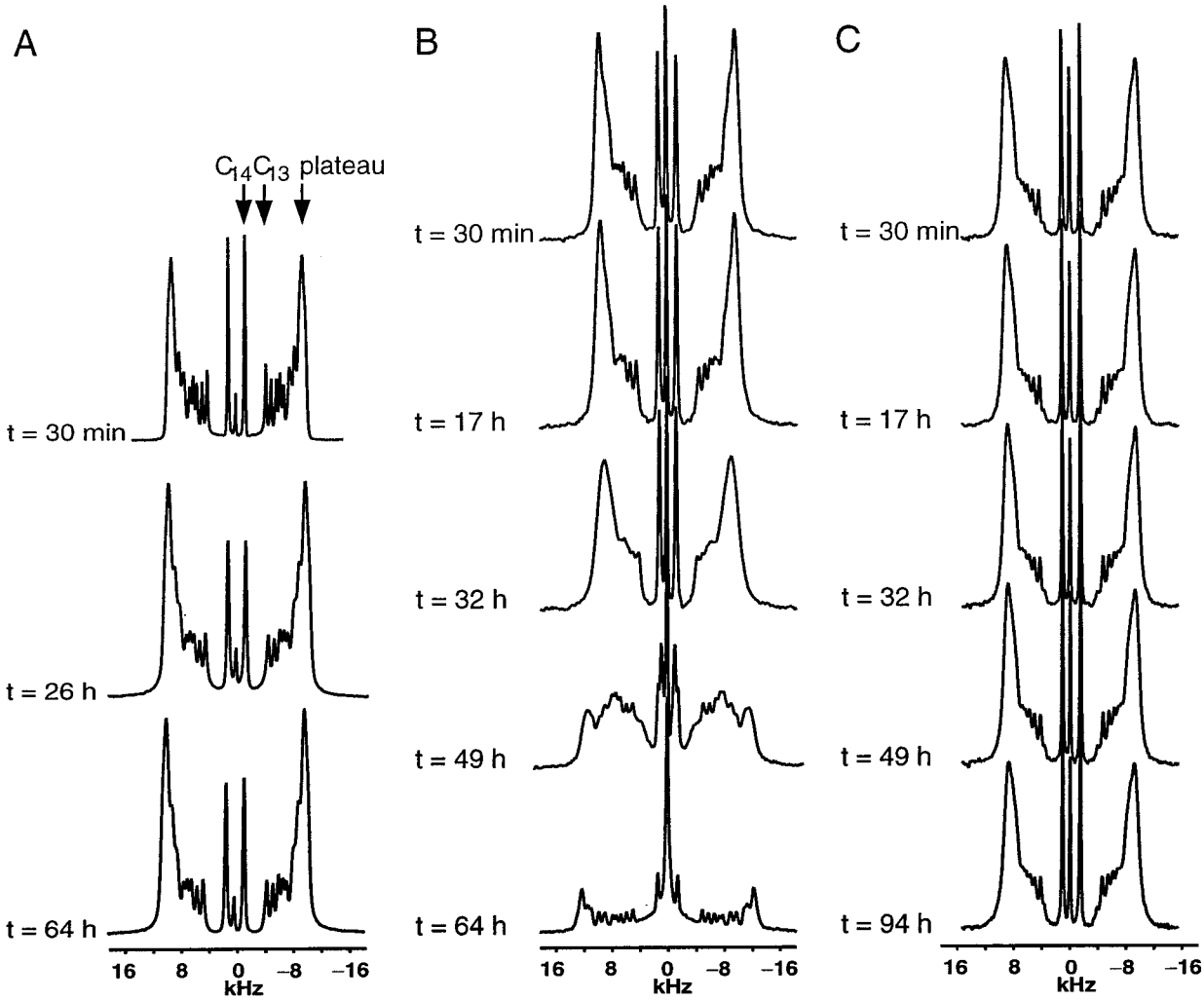

FIGURE 1 Time evolution of the 55.3 MHz deuterium quadrupole echo spectra of freshly prepared bicellar solutions containing 20% (w/w) DMPC, DMPS, and DHPC with $r = 3.0$ and $q = 3.2$ in deuterium-depleted water. The spectra were obtained at 40°C at the indicated times after sample preparation. The samples in *A* and *B* were nominally identical; the pH was adjusted to 4.7 (ambient pH). The only difference was the presence of small amounts of, respectively, chain-perdeuterated DMPC (DMPC-d_{54}) in sample *A* and chain-perdeuterated DMPS (DMPS-d_{54}) in sample *B*. Sample *C* was nominally identical to sample *B*, except that the pH was adjusted to 5.5. The assignments of doublets to the terminal methyl group (C_{14}), the adjacent methylene group (C_{13}), and the five methylene groups (plateau) closest to the phospholipid headgroup are indicated with small arrows. Spectra of DMPG bicelles were almost identical.

during which time the samples were repeatedly stored at −20°C and thawed for each experiment, and the splittings were found to be reproducible within ±3%. At lower temperatures, between 25°C and 30°C, a small but consistent increase in ordering of the myristoyl chains was observed in the acidic bicelles that was not previously observed for neutral bicelles (data not shown). This could be due to the higher viscosity of these samples compared to neutral bicelles.

The effect of temperature on the quadrupolar splittings of the plateau and the terminal CD_3 group is illustrated in Fig. 3. In this plot the observed splittings have been normalized to the splittings observed at 34.2°, the lowest temperature explored in the nematic phase. The temperature dependence of the normalized ^2H splittings, $\delta\Delta_i(T)$, of the acidic bicelles is 38% steeper than for neutral bicelles, i.e., $\Delta\delta\Delta_{plateau}/\Delta T)_{acidic} = -0.0137$ with a linear regression of 0.96 as compared to $(\Delta\delta\Delta_{plateau}/\Delta T)_{neutral} = -0.0099$ with a linear regression of 0.91. The plateau splittings in particular clearly show a stronger temperature dependence for acidic bicelles than for neutral bicelles. In contrast, the $\delta\Delta_i(T)$ close to the center of the hydrophobic layer was similar for the two types of bicelles, and the temperature dependence ranges between 0.024 and 0.026 with correlations larger than 97%. The fact that differences between acidic and neutral bicelles were only observed near the headgroups may be due to electrostatic effects, such as coulombic interactions between phospholipid molecules in the interface.

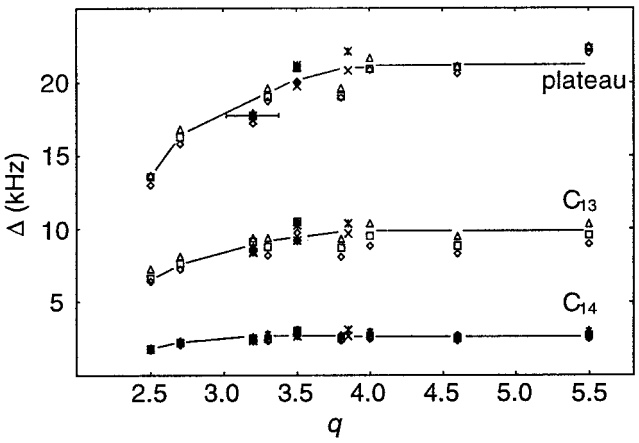

FIGURE 2 Deuterium quadrupole splittings (Δ) observed for neutral (*open symbols*) and DMPS (*solid symbols*) or DMPG (*crosses*) bicelles as a function of q for three distinct positions (C_{14}, C_{13}, and the plateau region) along the myristoyl chains. The spectra were obtained at pH 5.5 and at three temperatures: 34.2°C (Δ, Δ, no data for DMPG), 36.2°C (| |, | |, *), and 39°C (◇, ◇, x). Three regions of the spectra corresponding to different regions of the lipid chain were analyzed: the C_{2-5} methylenes (the "plateau"), the C_{13} methylene, and the C_{14} methyl groups. The horizontal error bar represents an estimate of the uncertainty in q.

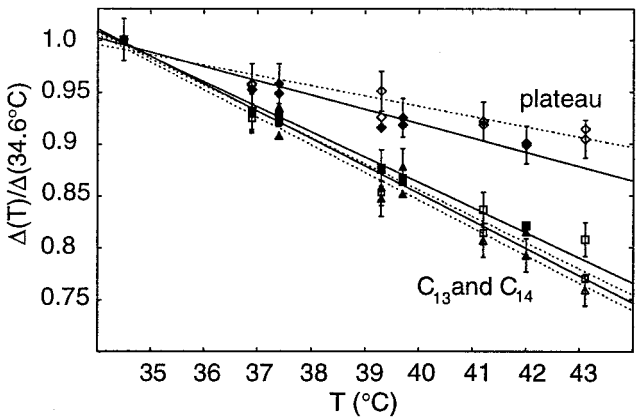

FIGURE 3 Temperature dependence observed for selected normalized quadrupolar splittings in the ^{2}H NMR spectra of DMPX-d_{54} (X = G, S, or C) and DMPC-d_{54} in neutral or acidic phospholipid bicelles. The splittings for the C_{14} deuterons (*squares*), the C_{13} deuterons (*triangles*), and the plateau region (C_2–C_6, *diamonds*) were normalized separately to those observed at 34.2°C. The data from neutral bicelles (*open symbols*) and bicelles with acidic phospholipids (*solid symbols*) were fit separately to linear functions. Four independent sets of data for $q = 3.5$ bicelles were used, two with DMPS ($r = 3.0$) and two with DMPG ($r = 3.0$) as the anionic component, and two sets of data with $q = 3.2$ and 3.5 were used for neutral DMPC/DHPC bicelles. The slope for the plateau region of the data for bicelles with acidic phospholipids (*solid line*) was 38% steeper than for the neutral bicelle data (*dashed line*), which is significantly larger than the error based on linear regression, which gave ±20% by standard error propagation. No significant difference was observed for the groups at the end of the myristoyl chain. For simplicity data of DMPG and DMPS are presented with the same symbols, as there was no significant difference observable.

The pH dependence of the quadrupolar splittings for $q = 3.2$ bicelles with 25% DMPS was measured on fresh samples analyzed within the first 24 h. The plateau splittings decreased by 20% between pH 4.5 and 6.0, in the region of the serine carboxyl group pK_a of 5.5. The decrease in splittings was smaller (10%) and more gradual for the terminal CD_2 and CD_3 groups. We ascribe the decrease in the plateau splittings to a decrease in the lateral pressure of the bilayer brought about by an increase in the electrostatic repulsion between the charged headgroups, which is known to reduce the liquid crystalline ordering (Cevc and Marsh, 1987).

Changing the ionic strength affects neutral and acidic bicelles differently. Addition of KCl to neutral bicelles at 40°C and pH 5.5 had no effect on the ordering of neutral bicelles up to 1 M concentration (Fig. 4). Above 1 M, the plateau splitting increased considerably, whereas the methyl splitting remained almost constant. This observed increase in order near the lipid-water interface may be attributed to salt-induced dehydration (Cevc and Marsh, 1987), which affects the lamellar order S_{lipid} and the lipid organization in the bilayer rather than the bicellar order S_{nn}. In acidic bicelles with 25% DMPS, the plateau and methyl splittings both decreased by 5–7% between 0.1 M and 1 M, and above 1 M alignment was not achieved at all. This observed decrease in ordering may be due to an effect on the bilayer system, S_{lipid}, or on the discotic system, S_{nn}. The observed decrease in order is inconsistent with Gouy-Chapman theory (Träuble, 1976; Cevc and Marsh, 1987), which would predict an increase in S_{lipid} due to an increase in lateral pressure with increasing ionic strength (Träuble, 1976; Träuble et al., 1976; Jähnig, 1976; Cevc et al., 1981; Marsh, 1990). Rather than interactions between phospholipid molecules, it is possible that the decrease in splitting results from interactions between individual bicelles, and reduction of the orientation potential (Maier and Saupe, 1958). At higher ionic strength (2.9 M KCl), where the prediction of the Gouy-Chapman theory fails (Cevc et al., 1981), no alignment can be obtained and the acidic bicelle sample becomes visibly turbid. This observation is indicative of the formation of larger lipid structures. The salt-induced dehydration and protonation of the interface seem to induce strong interactions between bicelles as well as in the bilayers that lead to the destruction of bicelles and different forms of aggregation in such lyotropic systems (Boggs, 1984). The steric effect of large cations appears to influence the lipid order in the bicelles rather than the bicellar order. At physiological conditions (~100 mM salt), increasing the size of the cation by using tetramethylammonium leads to a measurable decrease in the splittings at constant pH (data not shown). More reliable separation of the underlying effects will require additional information from spin relaxation experiments.

Bivalent ions, such as Ca^{2+}, that form complexes with DMPS (Holwerda et al., 1981; Hinderleiter et al., 1994),

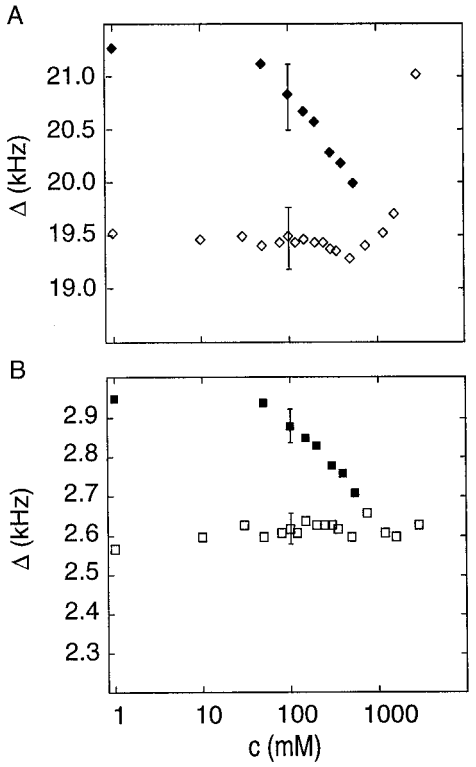

FIGURE 4 Quadrupolar splittings for the terminal methyl groups (C_{14}, *squares*) and the plateau region (C_2–C_6, *diamonds*) observed at 40°C and pH 5.5 as a function of KCl concentration for bicelles containing neutral (*open symbols*) and acidic (*solid symbols*) ($q = 3.5$, $r = 3.0$) phospholipids at 20% (w/w) total phospholipid concentration. Above 1 M KCl the bicelles containing acidic lipids were completely destroyed; macroscopic order was lost, the samples became opaque, and only powder patterns were observed for DMPC-d_{54}. The dependence of bicellar ordering on ionic strength was weak for concentrations below 50 mM for bicelles containing neutral (*triangles*) and acidic (*squares*) phospholipids. With increasing salt concentration, the plateau (*A*) and methyl (*B*) splittings remained constant for neutral bicelles up to 800 mM salt and decayed for bicelles containing acidic phospholipids.

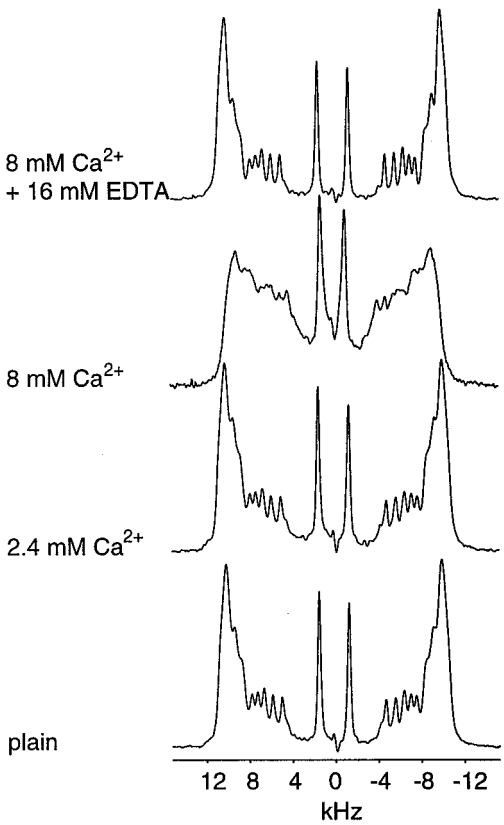

FIGURE 5 Deuterium quadrupole echo spectra recorded at 40°C and pH 5.5 for $r = 3.0$ 20% (w/w) bicelles containing DMPS-d_{54} (r = [DMPC]/[DMPS-d_{54}] = 3). The addition of 2.4 mM $CaCl_2$ had little effect on the order, while at $[Ca^{2+}] = 8$ mM a white precipitate formed that contributed a large powder pattern to the spectrum. The addition of excess EDTA (16 mM) reversed the phase separation: the sample became transparent and well aligned, and within experimental error (± 0.1 kHz) the splittings were identical to those observed for the original sample before the addition of Ca^{2+} ions.

show a similar but amplified behavior compared to K^+. No measurable effect on the bicelle ordering was observed for $[Ca^{2+}] < 100$ mM in neutral bicelles, and only a slight decrease in order was observed at 100 mM Ca^{2+}. For acidic phospholipids, however, it was not possible to align the bicelle system at even 8 mM Ca^{2+}, as shown by the observed powder-like ^2H NMR spectrum (Fig. 5). The sample also appeared turbid, suggesting the formation of a larger lipid aggregate, probably due to $Ca(DMPS)_2$ complex formation (Hinderleiter et al., 1994). Fig. 5 shows also that this effect was reversible, if the Ca^{2+} ions were removed from the headgroups by the addition of EDTA.

Complex formation of phospholipid molecules is also known to be induced by trivalent lanthanide ions (Hauser and Phillips, 1979) but depends on the amount of lanthanide ions with respect to phospholipid ions (Chrezeszczyk et al., 1981; Petersheim et al., 1989; Sun and Petersheim, 1990). Indeed, to "flip" acidic bicelles (Prosser et al., 1998) while maintaining well-ordered structures, only small concentrations of lanthanides can be added. We chose to work with Tm^{3+} because it has the largest anisotropy matrix element (Bleaney, 1972; Prosser et al., 1998). A positively aligned lyotropic lamellar phase for bicelles with q = 3.5 was achieved by the addition of 2 mM Tm^{3+} ions (Fig. 6).

Interaction of myristoylated peptides with acidic bicelles

To test the use of acidic bicelles in studies of membrane-associating peptides we recorded the ^2H NMR spectra of two samples containing 20% (w/w) lipid and the myristoylated 14-residue N-terminal peptide Myr-d_{27}-GSSKSKPKDPSQRR from the tyrosine kinase pp60(*v-src*) (Resh, 1990). The samples were prepared as described previously for another myristoylated peptide (Struppe et al., 1998), and one sample contained neutral $q = 3.5$ DMPC/

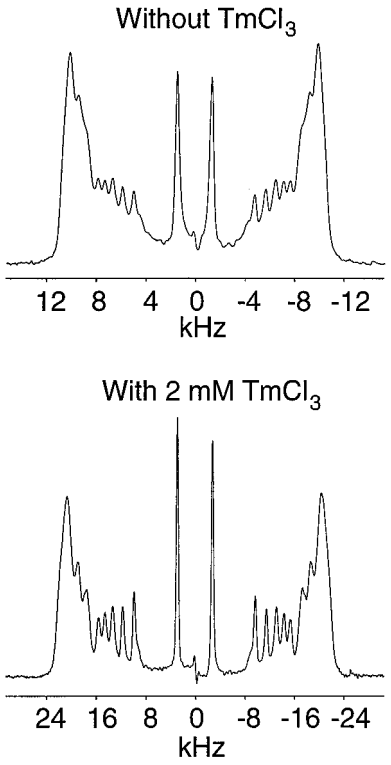

FIGURE 6 Deuterium quadrupole echo spectra of a 20% (w/w), $q = 3.5$, r = [DMPC]/[DMPS-d_{54}] = 3 bicellar solution recorded at pH 5.5 and 40°C before and after the addition of 2 mM $TmCl_3$. The spectrum (*A*) without lanthanide ions has been expanded horizontally by a factor of 2 relative to that (*B*) containing Tm^{3+} ions to facilitate the comparison of negatively (*A*) and positively (*B*) ordered bicelles.

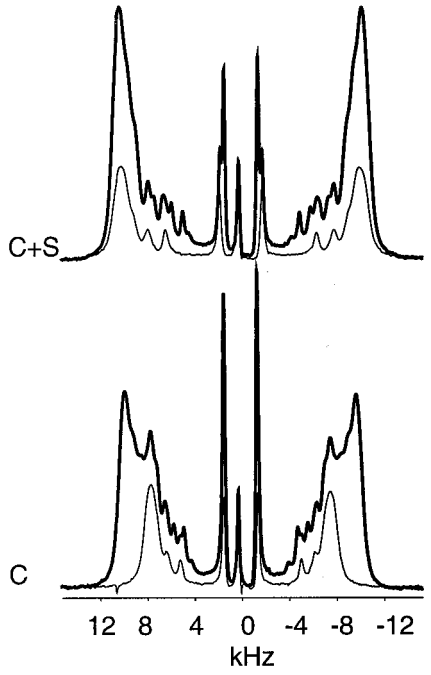

FIGURE 7 Deuterium quadrupole echo NMR spectra of the N-terminal 14-residue peptide Myr-d_{27}-GSSKSKPKDPSQRR of pp60(*v-src*) incorporated into the bilayer of $q = 3.5$ DMPC/DHPC (C) and DMPC/DMPS/DHPC (C + S) bicelles. Details of the sample preparation are given in Materials and Methods. The thinner line spectra are of bicelles containing the pp60(*v-src*) peptide with deuterated myristol chains, and only the myristoyl group deuterons are observed. The thicker line spectra are for the same sample as the thinner line, except that a small additional amount of bicelles containing DMPC-d_{54} was added and allowed to equilibrate, so that the deuterium signal from the phospholipids in the presence of peptide could be observed. The "extra" resonance (an unresolved doublet) near the center of the spectra arises from HOD.

DHPC bicelles, the other $q = 3.5$, $r = 0.25$ DMPC/DMPS/DHPS bicelles. Both samples were prepared with a 0.033 molar ratio of peptide to long-chain phospholipid, and both sets of spectra were run at pH 5.5 and 40°C. The deuterium quadrupole echo spectra of the two samples are presented in Fig. 7. Spectra of samples in which only the peptide myristoyl group was deuterated were recorded first. Afterward, a small amount of $q = 3.5$ bicellar solution containing DMPC-d_{54} was added to the samples, and the resulting spectra are plotted with thick lines. Comparison of the spectra of the myristoylated peptide in bicelles made of neutral phospholipids (C) compared to bicelles made of a mixture of neutral and acidic phospholipids (C + S) shows that the DMPC splittings were not reduced significantly by the addition of peptide to the samples, indicating that bicelle integrity is maintained. In the neutral bicelles, the ^2H quadrupolar splitting associated with the terminal peptide myristoyl methyl group was similar to the DMPC methyl splittings, while in bicelles containing acidic lipids the peptide myristoyl methyl splitting was actually larger by 24% than that arising from the DMPC methyl groups. In neutral bicelles, the splitting from the myristoyl CD_2 groups closest to the peptide N-terminal glycine was smaller than the lipid plateau splitting by a factor $\Delta_P/\Delta_L = 0.78 \pm 0.02$. In bicelles with acidic lipids, on the other hand, the myristoyl methylene splittings were similar to the phospholipid plateau splittings ($\Delta_P/\Delta_L = 0.98 \pm 0.02$). The actual splittings are given in the caption to Fig. 7.

It is interesting to compare these observations on this positively charged myristoylated pp60(*v-src*) peptide in bicelles containing either neutral or acidic lipids with similar observations made for the interaction of the uncharged myristoylated peptide Myr-GNAAAAKKGSEQES from protein kinase A (PKA peptide) (Struppe et al., 1998). Whereas in bicelles containing acidic lipids the pp60(*v-src*) peptide myristoyl methyl splitting was actually larger by 24% than that arising from the DMPC methyl groups, the methyl group of the myristoyl chain of the PKA peptide was similarly ordered as compared to the methyl groups of the lipid chains. This result suggests that the pp60(*v-src*) peptide myristoyl group is not inserted fully into the hydrophobic core of the acidic bicelle lipid bilayer because the order of the lipid chains actually decreases in going from the plateau to the core of the hydrophobic bilayer where the methyl groups are located.

For the neutral PKA peptide, although the methyl group of the myrstoyl chain had an order similar to that of the lipid methyl groups, the plateau region of the myristoyl group (Δ_P) was significantly less ordered than the plateau region of the lipids (Δ_L). Indeed, for the DMPS-containing bicelle samples $\Delta_P/\Delta_L = 0.69$ for the plateau splitting. We rationalized these observations by invoking a kink in the myristoyl chain of the peptide, which allows the outer part of the myristoyl chain to wobble. Furthermore, the plateau splitting ratio for the PKA peptide myristoyl group only increased from 0.64 to 0.75 in going from neutral to acidic bicelles, indicating that neither the peptide nor the myristoyl group interacted significantly with the acidic lipids. For the pp60(*v-src*) peptide, the plateau splitting ratio Δ_P/Δ_L was 0.78 in neutral bicelles and increased to 0.98 in acidic bicelles. This large increase in plateau region order observed for the myristoyl group of the pp60(*v-src*) peptide in the acidic bicelles supports the notion that lysines and/or arginines in a "basic patch" on the peptide may specifically interact with the negatively charged phosphatidylserine or phosphatidylglycerol headgroups.

CONCLUSIONS

Bicelle solutions containing 25% acidic phospholipids such as DMPS and DMPG have been prepared and are stable at and above pH 5.5. The solutions are relatively easy to prepare, and the presence of the acidic phospholipid does not interfere with the overall bicellar ordering. Bicelles containing acidic phospholipids provide a more biologically relevant membrane mimetic and can be prepared under a variety of conditions, including pH, temperature, and salt concentration, all of which are important in studies of biological systems. The potential use of neutral and acidic bicelles as a means of characterizing peptide-membrane interaction has been demonstrated by the results obtained for the N-terminal myristoylated 14-residue segment of pp60(*v-src*), which has a net charge of +4. The degree of ordering observed for the peptide myristoyl chain is reduced from that of the phospholipids themselves in neutral bicelles, but similar to that of the phospholipids in acidic bicelles. Furthermore, the degree of ordering of the peptide myristoyl chain in acidic bicelles was significantly higher than that observed for the myristoyl chain of a neutral peptide derived from protein kinase A. Presumably the additional ordering is the result of stabilizing electrostatic interactions between the five basic residues of the pp60(*v-src*) peptide and the carboxyl group of DMPS. Clarification of this issue is being sought in high-resolution proton NMR studies of the pp60(*v-src*) peptide in isotropic $q = 0.5$ neutral and acidic bicellar solution. High-resolution proton NMR could also give insights into intermolecular NOEs that might be indicative of partitioning of the peptide into "rafts" of acidic phospholipids.

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