

Structural Transitions in Short-Chain Lipid Assemblies Studied by ^{31}P -NMR Spectroscopy

Jörg H. Kleinschmidt*[†] and Lukas K. Tamm*

*Department of Molecular Physiology and Biological Physics, University of Virginia Health System, Charlottesville, Virginia 22908-0736, and [†]Fachbereich Biologie, Universität Konstanz, D-78457 Konstanz, Germany

ABSTRACT The self-assembled supramolecular structures of diacylphosphatidylcholine ($diC_n\text{PC}$), diacylphosphatidylethanolamine ($diC_n\text{PE}$), diacylphosphatidylglycerol ($diC_n\text{PG}$), and diacylphosphatidylserine ($diC_n\text{PS}$) were investigated by ^{31}P nuclear magnetic resonance (NMR) spectroscopy as a function of the hydrophobic acyl chain length. Short-chain homologs of these lipids formed micelles, and longer-chain homologs formed bilayers. The shortest acyl chain lengths that supported bilayer structures depended on the headgroup of the lipids. They increased in the order $\text{PE} (C_6) < \text{PC} (C_9) \leq \text{PS} (C_9 \text{ or } C_{10}) < \text{PG} (C_{11} \text{ or } C_{12})$. This order correlated with the effective headgroup area, which is a function of the physical size, charge, hydration, and hydrogen-bonding capacity of the four headgroups. Electrostatic screening of the headgroup charge with NaCl reduced the effective headgroup area of PS and PG and thereby decreased the micelle-to-bilayer transition of these lipid classes to shorter chain lengths. The experimentally determined supramolecular structures were compared to the assembly states predicted by packing constraints that were calculated from the hydrocarbon-chain volume and effective headgroup area of each lipid. The model accurately predicted the chain-length threshold for bilayer formation if the relative displacement of the acyl chains of the phospholipid were taken into account. The model also predicted cylindrical rather than spherical micelles for all four diacylphospholipid classes and the ^{31}P -NMR spectra provided evidence for a tubular network that appeared as an intermediate phase at the micelle-to-bilayer transition. The free energy of micellization per methylene group was independent of the structure of the supramolecular assembly, but was -0.95 kJ/mol (-0.23 kcal/mol) for the PGs compared to -2.5 kJ/mol (-0.60 kcal/mol) for the PCs. The integral membrane protein OmpA did not change the bilayer structure of thin ($diC_{10}\text{PC}$) bilayers.

INTRODUCTION

The properties of phospholipids as the major components of biological membranes have been the subject of extensive biochemical and biophysical research for several decades. Much insight has been gained on their structure, function, and physical properties in lipid bilayers. Although cells produce a relatively small number of different lipid headgroups (lipids with different headgroups each constitute a different lipid class), membrane phospholipids exhibit a very large array of different hydrophobic acyl chains. Most studies of phospholipids focused on lipids with C_{14} or longer chains, which are the most frequently found species in cell membranes (see, for example, Morein et al., 1996). The hydrophobic chain length of phospholipids controls the thickness of the bilayers that are formed from them and thereby may control the activity of integral membrane proteins. For example, the hydrophobic thickness of the bilayer regulates the activity of the ion channel gramicidin A (Galbraith and Wallace, 1998) and the activity of the Ca-ATPase and other membrane transporters depends on the acyl chain length of the phospholipid bilayer in which it resides (Cor-

nea and Thomas, 1994; Dumas et al., 2000). Recently, we observed that the folding and membrane insertion of the outer membrane protein A (OmpA) of *Escherichia coli* depends on the thickness of the lipid bilayer (J. H. Kleinschmidt and L. K. Tamm, in preparation). The folding of OmpA was greatly facilitated by thin membranes and also by short-chain phospholipid micelles. Short-chain phospholipids with ten or fewer carbons are found as oxidation products in the human plasma (Schlame et al., 1996). Some short-chain lipids act as specific inhibitors of membrane proteins. For example, $diC_{10}\text{PC}$ inhibits the activity of the apical membrane amiloride-sensitive Na channel (Ropke et al., 1997). Some micelle-forming diacylphospholipids (especially $diC_6\text{PC}$) have been characterized as excellent activity-preserving detergents for membrane protein extraction and reconstitution (Hauser, 2000; Kessi et al., 1994). Micelles of $diC_6\text{PC}$ have also been used as suitable environments for high-resolution nuclear magnetic resonance (NMR) studies of membrane proteins (Fernández et al., 2001; Marassi et al., 1999).

In all these studies involving relatively short-chain phospholipids, it is of interest to know what supramolecular structures the phospholipid assemblies form in aqueous solutions of the relevant buffer and salt composition. A survey of the literature revealed that the acyl chain-length threshold beyond which phospholipid classes form bilayers rather than micelles has not been well documented. Tausk et al. (1974a,b,c) determined the critical micell concentrations (CMCs) (see Note 1) of the $diC_n\text{PC}$ homolog series from $n = 6-9$. Although these authors report the average aggre-

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Address reprint requests to Dr. Jörg H. Kleinschmidt at Fachbereich Biologie, Universität Konstanz, Universitätsstrasse 10, D-78457 Konstanz, Germany. Tel.: +49-7531-883360; Fax: +49-7531-883183; E-Mail: joerg.helmut.kleinschmidt@uni-konstanz.de.

Abbreviations used: ($diC_n\text{PC}$), diacylphosphatidylcholine; ($diC_n\text{PE}$), diacylphosphatidylethanolamine; ($diC_n\text{PG}$), diacylphosphatidylglycerol; ($diC_n\text{PS}$), diacylphosphatidylserine.

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gation numbers (molecular weights) of the micelles formed by these lipids, they did not explicitly investigate the supramolecular structures into which each of these lipids assemble. Eastoe et al. (1998) reported that *diC*₇PC formed cylindrical micelles. No comparable data appears to be available for the other diacylphospholipids.

In the present study, we have used solid-state ³¹P-NMR spectroscopy to determine the minimum chain lengths that are required for the formation of lipid bilayers for the homologous series of *diC*_nPC, *diC*_nPE, *diC*_nPG, and *diC*_nPS. We have also investigated the effect of the ionic strength on the supramolecular structures that are formed by these lipids. Our results can be rationalized with the geometric packing model of Israelachvili et al. (1976, 1977) if the relative displacement of the acyl chains in phospholipids is taken into account.

MATERIALS AND METHODS

Materials

*DiC*_nPC, *diC*_nPE, *diC*_nPG, and *diC*_nPS with chain lengths of $n = 6$ –14 carbon atoms were purchased from Avanti (Alabaster, AL). Coomassie Brilliant Blue R-250 was from Biorad (Hercules, California).

Sample preparation for ³¹P-NMR spectroscopy

Lipid samples

Lipids were dissolved in chloroform or in a 1:1 mixture of chloroform and methanol. The solvent was evaporated under a stream of nitrogen, and the dry lipid films were hydrated to a concentration of 25 mg lipid in 200 μ l 10 mM borate buffer, pH 10, containing 2 mM EDTA or in distilled water. In most cases, the same results were obtained in this buffer and water. Phosphatidylethanolamines were hydrated in borate buffer, pH 8.5, to avoid deprotonation of the ethanolamine headgroup. The hydrated samples were homogenized by vortexing and repeated freeze–thawing in liquid nitrogen and a water bath at 30°C.

Lipid samples with incorporated OmpA

One hundred microliters of a 3.15 mM solution of denatured OmpA in borate buffer, pH 10, containing 2 mM EDTA and 8 M urea, was refolded by mixing with 900 μ l of a 31.6 mM solution of *diC*₁₀PC (16 mg lipid) in the same buffer without urea. The mixture was incubated overnight at room temperature to yield quantitatively refolded OmpA in *diC*₁₀PC (manuscript in preparation). The lipid–protein complexes were concentrated to a final volume of 200 μ l using centrifuge concentrators in an Eppendorf Centrifuge 5415C and then transferred into NMR tubes. The lipid/protein ratio of the sample was 90 mol/mol.

³¹P-NMR spectroscopy

³¹P-NMR spectra were recorded at 146.15 MHz in the magnetic resonance laboratory of the Department of Chemistry at the University of Virginia. The spectrometer consisted of an Oxford Instruments 8.45 T magnet and primarily Tecmag (Houston, TX), Doty Scientific (500A 8–150 MHz rf amplifier) and Henry Radio (2002A 360 MHz rf amplifier), and Herley-AMT (Anaheim, CA) components. MacNMR (Tecmag) was used to acquire and process spectra. The phase-cycled Hahn echo pulse sequence

(Rance and Byrd, 1983) was used. The 90° pulse width was 8 μ s, the delay between pulses was 50 μ s, and the sweep width was 100 kHz. Continuous-wave ¹H-decoupling was used during data acquisition and the decoupling field strength ($\gamma H_2/2\pi$) was 35 kHz. The recycle time was 2 s. Up to 45,000 scans were accumulated. Free-induction decays were apodized with 50-Hz exponential line broadening, zero-filled twice to 16,384 points and Fourier transformed. All spectra were recorded at 25°C.

Determination of critical micelle concentrations

CMCs were estimated by a colorimetric assay as previously described (Kleinschmidt et al., 1999). The method utilizes a red shift of the absorption maximum of Coomassie Brilliant Blue R-250 from 555 nm in the absence to 595 nm in the presence of detergent micelles. 10 μ l of a 10-mM solution of Coomassie blue in borate buffer were added to 1-ml solutions containing step-wise increased concentrations of detergent. UV spectra from 500 to 650 nm were recorded on a Hitachi UV spectrometer. Background spectra without Coomassie blue were subtracted.

RESULTS

³¹P-NMR spectra of short-chain phospholipids

Figure 1 shows proton-decoupled ³¹P-NMR spectra of *diC*_nPC, *diC*_nPE, *diC*_nPG, and *diC*_nPS with saturated acyl chains ranging in length from 6 to 14 carbon atoms. Aqueous dispersions of *diC*_nPE show anisotropic spectra that are characteristic for lamellar lipid phases at all acyl chain lengths. The spectra up to *diC*₁₂PE are axially symmetric, indicating rapid rotational averaging of the chemical shift tensor around the membrane normal, which is characteristic for bilayers in the liquid–crystalline phase (Seelig, 1978). The spectrum of *diC*₁₄PE is characterized by a broadened intrinsic line width, which is expected for this lipid in the gel phase. The spectra of the other lipid species display shapes that depend critically on the acyl chain length of the lipid. As the chain lengths were increased, the spectral lineshape changed from a narrow isotropic peak to an anisotropic lineshape characteristic for axially symmetric lamellar lipid phases (see Note 2). For the short-chain lipids used here, the isotropic lineshape indicates that the lipids are assembled in micelles that tumble rapidly and isotropically on the NMR time scale (Cullis and de Kruijff, 1979). The change in the supramolecular structure from micelles to lipid bilayers occurred at different chain lengths in the four lipid classes that we investigated. The minimal chain lengths that were required to form a bilayer phase were C₉ for PC, C₉ or C₁₀ for PS, and C₁₁ or C₁₂ for PG. The spectrum of *diC*₁₀PG showed an isotropic signal superimposed on a highly narrowed anisotropic spectrum with a lineshape similar to lipids in inverted hexagonal (H_{II}) phases (Cullis and de Kruijff, 1979; Seelig, 1978), but with a narrower line width. Some lamellar-phase spectra of Fig. 1 show signs of isotropic signals at 0 ppm. These could arise from small amounts of small unilamellar lipid vesicles that coexist with the majority of multilamellar

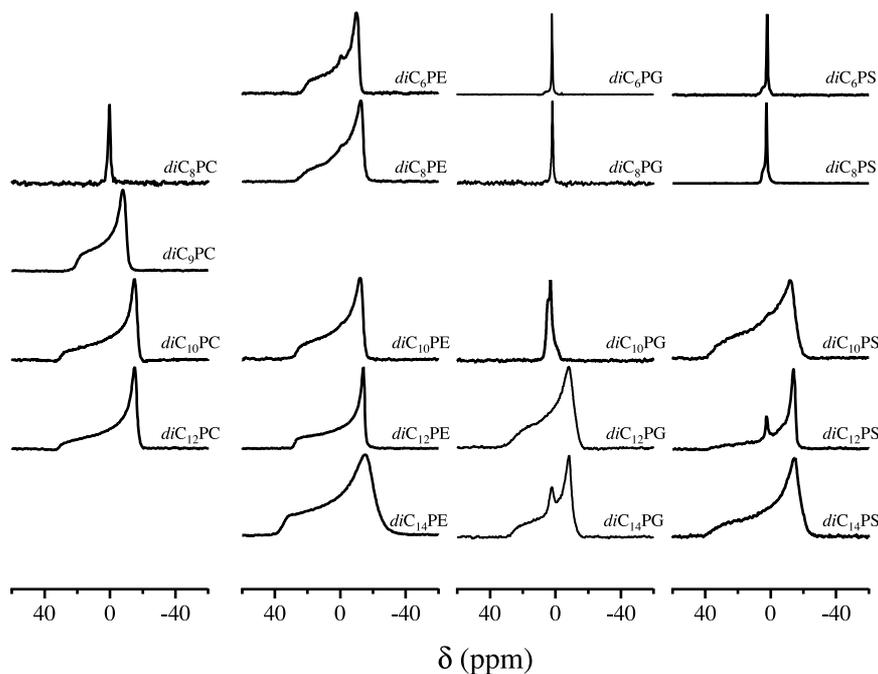


FIGURE 1 Proton-decoupled ^{31}P -NMR spectra of $diC_n\text{PCs}$, $diC_n\text{PEs}$, $diC_n\text{PGs}$, and $diC_n\text{PSs}$ of different acyl chain lengths. All spectra were recorded at lipid concentrations of 125 mg/mL and at 25°C. Aqueous dispersions of all lipids except $diC_n\text{PEs}$ were prepared in 10 mM borate buffer at pH 10. The pH of the borate buffer of the $diC_n\text{PE}$ samples was 8.5 because the pK of this lipid headgroup is 9.8. Very similar spectra were recorded in water and borate buffer for several of these lipids.

phases in these samples. Such vesicles may form during the repeated freeze–thaw cycles/vortexing and would undergo more rapid isotropic motions leading to narrow lines.

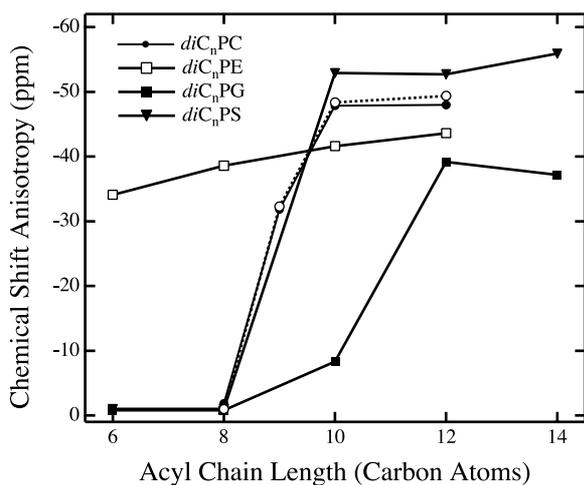
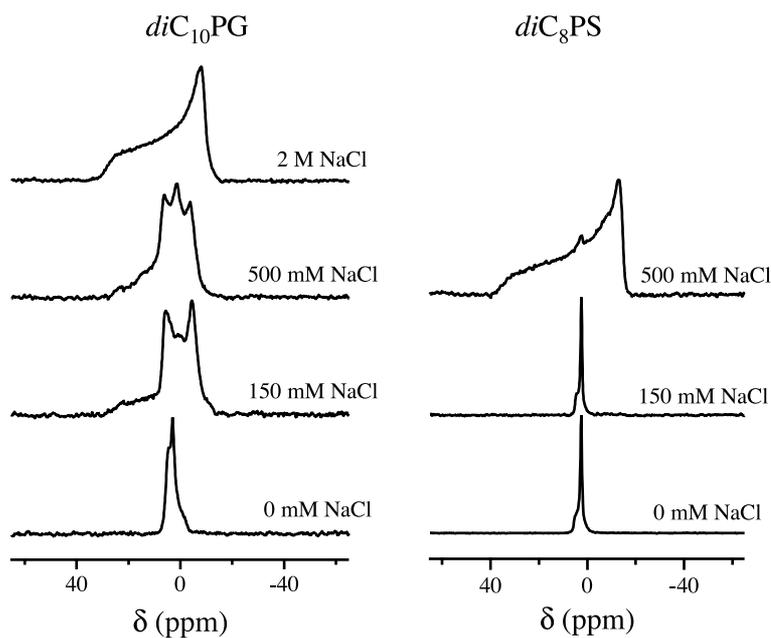


FIGURE 2 Chemical shift anisotropies of $diC_n\text{PCs}$ (circles), $diC_n\text{PEs}$ (open squares), $diC_n\text{PGs}$ (filled squares), and $diC_n\text{PSs}$ (triangles) as a function of the hydrophobic chain length. The CSAs were measured from the difference of the parallel (low-intensity) and perpendicular (high-intensity) edges of the ^{31}P -NMR spectra of Fig. 1 as described in Seelig et al. (1982). In addition, the CSAs of the ^{31}P -NMR spectra of $diC_n\text{PCs}$ dispersed in distilled water instead of buffer are plotted (open circles).

Chemical shift anisotropy of ^{31}P -NMR spectra

The chemical shift anisotropies (CSA) of the spectra of Fig. 1 are plotted as a function of the hydrophobic acyl chain length in Fig. 2. The absolute values of the CSA of the PEs increased by ~ 1.6 ppm for each added methylene segment in the acyl chains. The CSAs of the other phospholipids did not change much over the limited chain length range that produced lamellar liquid–crystalline phases of these lipids. The observed CSA values of the PEs, PCs, and PSs are in good agreement with published values of longer chain homologs of these lipids above their respective gel-to-liquid crystalline phase transition (Seelig and Seelig, 1980). The CSAs of $diC_{12}\text{PG}$ and $diC_{14}\text{PG}$ were 2–3 ppm smaller than the published value of $diC_{16}\text{PG}$ (Seelig and Seelig, 1980). When the CSAs of lipids of the same chain length with different headgroups are compared, e.g., all C_{12} species, one observes an increase of the CSA in the order, $\text{PG} < \text{PE} < \text{PC} < \text{PS}$. With the exception of PE, this order correlates with the increasing volumes of the lipid headgroups in this sequence. Because CSAs depend on headgroup order and headgroup orientation (Seelig, 1978), it is not straightforward to explain the observed effects. One would generally expect to observe more order (larger CSA) for larger headgroups, but order increases may be masked in measurements of CSA by changes of headgroup orientation that depends on the chemistry, hydration, hydrogen bonding, and charge interactions of each headgroup. The ability of PE head-

FIGURE 3 Proton-decoupled ^{31}P -NMR spectra of $diC_{10}\text{PG}$ and $diC_8\text{PS}$ obtained at different NaCl concentrations at 25°C. Both lipids show a transition from the micellar to the bilayer phase as the ionic strength is increased. $diC_{10}\text{PG}$ in 150 and 500 mM NaCl shows an additional spectral component that we interpret as representing a tubular lipid phase (see text).



groups to hydrogen-bond may be partially responsible for their larger than expected CSAs.

As noted above, the chain-length threshold for bilayer formation follows the different order of phospholipid classes, $\text{PE} < \text{PC} \leq \text{PS} < \text{PG}$. In addition to headgroup volume, headgroup charge appears to play an important role to explain the micelle-to-bilayer transition. Charge repulsion drives negatively charged PGs to the micellar phase and thereby moves this lipid species to the right end of the sequence. The primary amine-containing headgroups of PE and PS can form hydrogen bonds with the phosphate groups of neighboring lipids as demonstrated by crystallography (Hitchcock et al., 1974), infrared spectroscopy (Sen et al., 1988), and fluorescence spectroscopy (Shin et al. 1991; Slater et al., 1993). Hydrogen bonds can also be formed between the hydroxyl groups and the phosphate group of phosphatidylglycerol molecules (Zang et al., 1997), but the negative charge remains effective as a repulsive force between PG molecules. The hydrogen-bonding interactions in PE and PS reduce the effective headgroup area and thereby stabilize the bilayer over the micellar structure.

Stabilization of bilayer phase by screening of the surface charge

To test directly whether charge repulsion contributed to the stability of micellar phases of short-chain phospholipids, we measured ^{31}P -NMR spectra of $diC_{10}\text{PG}$ and $diC_8\text{PS}$ as a function of increasing salt concentration (Fig. 3). $diC_{10}\text{PG}$ was mostly micellar in the absence of added salt, but exhibited an axially symmetric bilayer spectrum in the presence of 2 M NaCl. The CSA in 2 M NaCl was -38 ppm, i.e., within 1 ppm of the CSAs of $diC_{12}\text{PG}$ and $diC_{14}\text{PG}$

without added salt. A complex phase behavior was observed with $diC_{10}\text{PG}$ in the presence of 150 and 500 mM NaCl. These spectra indicate the presence of three phases, i.e., isotropic micellar, lamellar, and a phase producing hexagonal phase-type spectra. The salt dependence of $diC_8\text{PS}$ was less complex. This lipid was in a micellar phase at ≤ 150 mM NaCl. 500 mM NaCl stabilized assemblies of $diC_8\text{PS}$ in the bilayer phase. The ^{31}P -NMR spectrum of this sample had a CSA of -51 ppm, i.e., it closely followed the trend of the longer-chain PS analogs in the absence of salt.

Effect of the integral membrane protein OmpA on the ^{31}P -NMR spectrum of $diC_{10}\text{PC}$

Some surface-active peptides such as melittin can cause micellization of phospholipid bilayers (Dempsey and Sternberg, 1991; Kleinschmidt et al., 1997), whereas large integral membrane proteins usually do not change the bilayer structure of most lipids. (Some transmembrane peptides transform lipid bilayer to inverted hexagonal phase structures (for a review, see Killian, 1998).) We were interested to find out whether an integral membrane protein could destabilize the bilayer phase of a marginally stable thin lipid bilayer. OmpA is a protein of the outer membrane of *E. coli*, where it forms a monomeric 8-stranded membrane-crossing β -barrel with a hydrophobic surface on its outer perimeter (Arora et al., 2001; Pautsch and Schulz, 2000). OmpA can be refolded from an unfolded state in solution into a membrane-inserted native state (Surrey and Jähnig, 1992; Kleinschmidt and Tamm, 1996; Arora et al., 2000). It therefore is a good model protein to examine the effect of protein insertion on the phase behavior of short-chain lipids. A proton-decoupled ^{31}P -NMR spectrum of OmpA that was

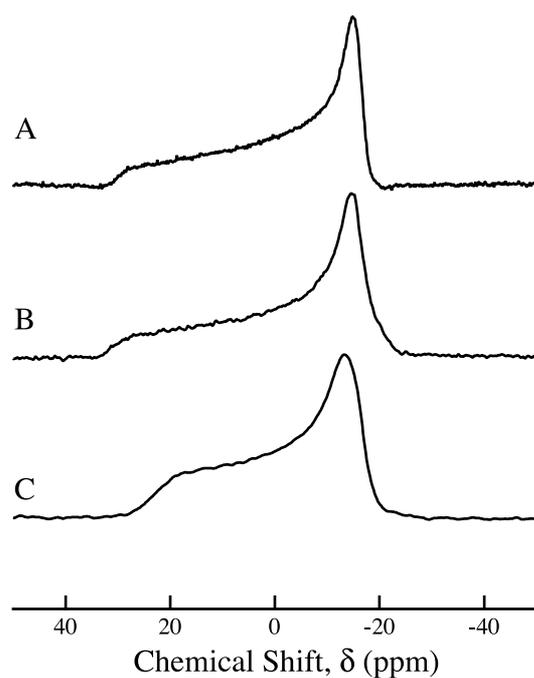


FIGURE 4 Effect of the integral membrane protein OmpA on ^{31}P -NMR spectra of $diC_{10}\text{PC}$ bilayers. Proton-decoupled ^{31}P -NMR spectra of (A) $diC_{10}\text{PC}$ bilayers in 10 mM borate buffer, pH 10, (B) $diC_{10}\text{PC}$ bilayers in borate buffer containing 800 mM urea, and (C) $diC_{10}\text{PC}$ bilayers containing refolded OmpA at a lipid/protein ratio of 90 (mol/mol) in borate buffer containing 800 mM urea were recorded at 25°C.

refolded into $diC_{10}\text{PC}$ bilayers at a lipid/protein ratio of 90 mol/mol is shown in Fig. 4 C. The shape of this spectrum indicates that the lipid remained in the lamellar phase. However, the CSA was reduced from 48 to 41 ppm (compare Fig 4, A and C). This change was caused by the protein and not by residual urea as demonstrated by spectrum B of the same lipid bilayers in 800 mM urea, but without OmpA. Similar (although smaller magnitude) reductions of the CSAs by integral membrane proteins have been reported previously for other integral membrane proteins in lipid bilayers (Yeagle and Romans, 1981; Tamm and Seelig, 1983). The proteins in these previous studies were reconstituted into bilayers of matching hydrophobic thickness by detergent methods.

CMCs of short-chain phosphatidylglycerols and phosphatidylserines

The increased chain-length threshold for bilayer formation of charged compared to zwitterionic phospholipids may also be reflected in smaller hydrophobic chain interaction energies at equal chain length. The free energy of hydrophobic interaction, ΔG_M^o , can be obtained from measurements of the CMCs of these lipids (Tanford, 1980),

$$\Delta G_M^o = \mu_M^o - \mu_W^o = RT \ln(X_{\text{CMC}}) + RT \ln(f_W), \quad (1)$$

TABLE 1 CMCs and free energies of micelle formation of PGs, PSs, and PCs

| Phospholipid | CMC (mM) | CMC (mole fraction units) | ΔG_M^o | |
|------------------------|----------|---------------------------|----------------|------------|
| | | | (kJ/mol) | (kcal/mol) |
| $diC_8\text{PG}$ | 1.21 | 2.2×10^{-6} | -26.3 | -6.3 |
| $diC_{10}\text{PG}$ | 0.42 | 7.6×10^{-6} | -28.9 | -6.9 |
| $diC_{12}\text{PG}$ | 0.13 | 2.3×10^{-6} | -31.9 | -7.6 |
| $diC_{14}\text{PG}$ | 0.011 | 2.1×10^{-7} | -37.8 | -9.0 |
| $diC_8\text{PS}$ | 2.282 | 4.1×10^{-5} | -24.8 | -5.9 |
| $diC_{10}\text{PS}$ | 0.096 | 1.7×10^{-6} | -32.5 | -7.8 |
| $diC_5\text{PC}$ | 90 | 1.6×10^{-3} | -15.8 | -3.8 |
| $diC_6\text{PC}$ | 15 | 2.7×10^{-4} | -20.2 | -4.8 |
| $diC_7\text{PC}$ | 1.4 | 2.5×10^{-5} | -26.0 | -6.2 |
| $diC_8\text{PC}$ | 0.27 | 4.9×10^{-6} | -30.0 | -7.2 |
| $diC_9\text{PC}$ | 0.029 | 5.2×10^{-7} | -35.5 | -8.5 |
| $diC_{10}\text{PC}$ | 0.005 | 9.0×10^{-8} | -39.8 | -9.5 |
| $lyso-C_{10}\text{PC}$ | 8 | 1.4×10^{-4} | -21.7 | -5.2 |
| $lyso-C_{12}\text{PC}$ | 0.9 | 1.6×10^{-5} | -32.7 | -6.5 |
| $lyso-C_{14}\text{PC}$ | 0.09 | 1.6×10^{-6} | -27.1 | -7.8 |

The data of PCs are from Tausk et al. (1974c), Helenius et al. (1979), Marsh (1990), and Heerklotz and Epanand (2001). Data of *lyso*-PCs are from Helenius et al. (1979).

where $\Delta\mu_M^o$ and $\Delta\mu_W^o$ are the chemical potentials of the lipid in micelles and water, respectively, X_{CMC} is the CMC in mole fraction units, and f_W is the activity coefficient of the monomeric lipid in water. The concentration of monomers is low, and it is therefore assumed that $f_W = 1$. Because the CMCs of short-chain charged phospholipids were not available in the literature, we determined the CMCs of the PG series using a colorimetric assay. We also determined the CMCs of PSs up to a chain length of 10 carbon atoms. Presumably due to differences in chain packing, the dye did not partition into bilayers of PSs with longer chain lengths and thereby precluded a determination of the CMCs of these lipids. The same problem arose when we attempted to measure the CMCs of the PE series. The measured CMCs are listed in Table 1 along with literature values of the CMCs of the PC series. The CMCs of the negatively charged lipids were four to eight times higher than those of the corresponding PCs. The absolute values of the derived free energies of micelle formation (Eq. 1) increase in the order, $\text{PG} \leq \text{PS} < \text{PC}$, i.e., the same order of headgroups that stabilize bilayers over micelles.

To find the increment of free energy of micelle formation per methylene segment, we plotted ΔG_M^o as a function of the acyl chain lengths (Fig. 5). The slopes of these plots are -4.9 kJ/mol (-1.2 kcal/mol) for PC and -1.9 kJ/mol (-0.46 kcal/mol) for PG. Both numbers represent the energy increment for two methylene segments (one on each chain). For a single methylene segment, these values are smaller (-2.5 kJ/mol for PC, and -0.95 kJ/mol for PG) than the -2.8 kJ/mol (-0.7 kcal/mol) per methylene segment found for the single-chain *lyso*- C_n PCs and other single-chain amphiphiles. This reduction is due to residual

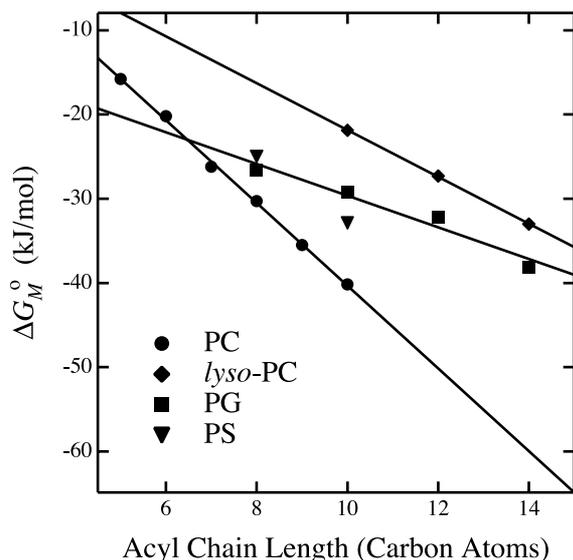


FIGURE 5 Free energies of micelle formation of several lipid classes as a function of the hydrophobic chain length at 25°C. All data are from Table 1.

hydrophobic interactions between the chains in two-chain molecules in the monomeric state. The reduced slope in Fig. 5 for the chain length dependence of ΔG_M^0 for the ionic PG compared to the zwitterionic PC is a result of the reduction of the ionic strength of the solution as the chain length is increased and is entirely consistent with previous observations of charged single-chain detergents at low ionic strength (Tanford, 1980). (The ionic strength of ionic detergent solutions in the absence of added salt is essentially equal to the CMC when the detergent is a 1:1 electrolyte.) Therefore, the PGs showed smaller apparent chain interaction energies per methylene segment.

DISCUSSION

In this study, we showed that phospholipids of different headgroup composition require different minimal chain lengths to assemble into bilayers rather than micelles. Israelachvili et al. (1976, 1977) developed a simple theory for the self-assembly of amphiphiles into micelles and bilayers. In this theory, the amphiphiles are approximated by rigid bodies of defined shapes. Entropy would generally drive the molecules into a large number of small micelles rather than into a small number of extended bilayers. However, the possibility to form micelles is limited by packing constraints. The number of phospholipids N that can pack into a spherical (S) or cylindrical micelle (C) is given by the surface of the lipid headgroup a , the radius of the micelle R , and the volume of the hydrophobic chains of the phospholipids v . For a cylindrical micelle of finite tube length L , the number of phospholipids is given by (Tanford, 1980):

$$N = \frac{\pi R_C^2 L + \frac{4}{3} \pi R_C^3}{v} = \frac{4\pi R_C^2 + 2\pi R_C L}{a}. \quad (2)$$

The radius of spherical (R_S) and long cylindrical micelles (R_C) can therefore easily be calculated if a and v are known. For spherical micelles ($L \rightarrow 0$), the radius is given by

$$R_S = 3 \frac{v}{a}. \quad (3)$$

For long cylindrical micelles ($L \gg R_C$), the terms $\frac{4}{3}\pi R_C^3$ and $4\pi R_C^2$ are negligible, and R_C is given by

$$R_C = 2 \frac{v}{a}. \quad (4)$$

If lipids of a given headgroup area and chain volume are to be packed into spherical or cylindrical micelles, the maximum extended hydrophobic chain length l must not be smaller than the radius of the micelle, to avoid empty space in the core of the micelle. Therefore, the micelle packing constraints must be

$$\frac{R_S}{l} = \frac{3v}{a \cdot l} \geq 1 \quad \text{and} \quad \frac{R_C}{l} = \frac{2v}{a \cdot l} \geq 1. \quad (5)$$

To our knowledge, this theory has previously only been tested for single-chain amphiphiles (Israelachvili et al., 1977). For diacylphospholipids with identical chains, l is given by the length of a single chain ($\sim 1.25 \text{ \AA}/\text{carbon atom}$, see legend to Fig. 6) plus the displacement of the two chains against each other. Extensive x-ray and NMR studies of several lipid classes indicate that the glycerol backbone in phospholipids continues linearly in the direction of the $sn-1$ chain and that the two chains are displaced by about three methylene segments relative to each other. This orientation of the phospholipid backbone relative to the chains is largely retained irrespective of whether the lipid is in a crystal, lipid bilayer, or detergent micelle (for a recent review, see Hauser, 2000). In micelles and fluid lipid bilayers, the orientation of the glycerol backbone must be regarded as a motionally averaged orientation, because the lipid molecules are undergoing various internal motions. Because the carbonyl carbon of the $sn-2$ chain is at the level of the C3 carbon of the glycerol backbone, the displacement between the chains is $\sim 4 \text{ \AA}$, as determined from the crystal structure of $diC_{14}PC$ (Fig. 6). This displacement was included in our calculations of the maximum hydrophobic chain length of diacylphospholipids.

To estimate the most likely supramolecular structures of the lipid assemblies, we calculated the radii of spherical and cylindrical micelles according to Eqs. 3 and 4 and compared these radii with the maximum extended lengths l of the hydrophobic chains of each lipid (Table 2). The ratios R_S/l and R_C/l listed in Table 2 indicate that the hydrophobic lengths of the lipids used in this study are too long to pack the lipids into spherical micelles, i.e., $R_S/l > 1.32$. However, the short-chain lipids that were characterized by isotropic ^{31}P -NMR spectra can be packed well into cylindrical mi-

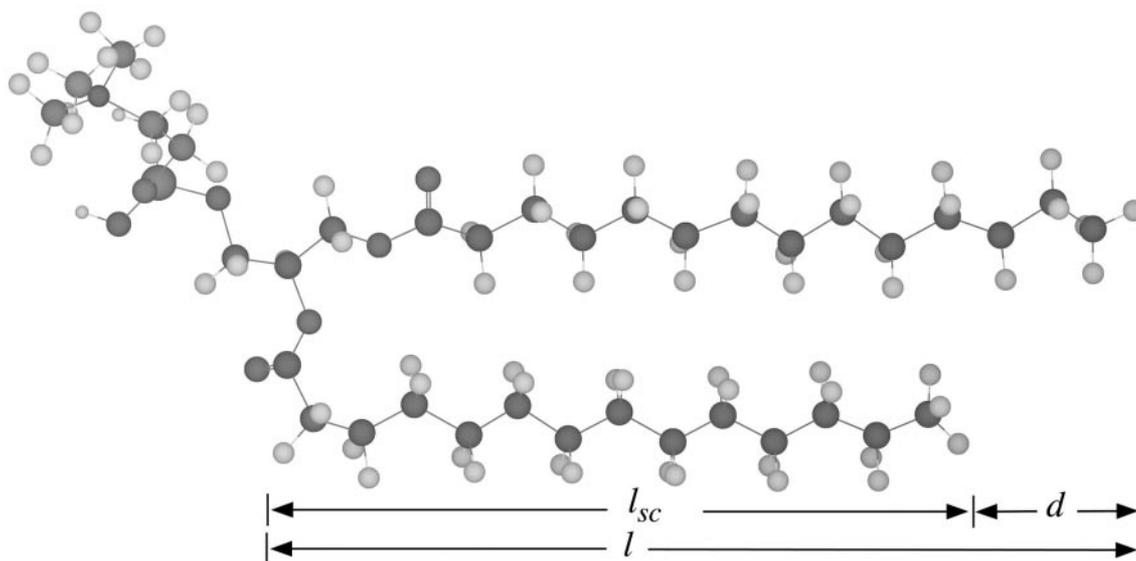


FIGURE 6 Crystal structure of $diC_{14}PC$ (Pearson and Pascher, 1979). The definitions for the calculations of the maximum extended chain lengths are indicated. $l = l_{sc} + d$. The length of the single chains in each phospholipid (l_{sc}) was calculated from the average length of a methylene segment in $diC_{14}PC$ (determined from the crystal structure) plus the length of a C–H bond in the terminal methyl group projected on the chain axis, i.e., for a chain with n carbons: $l_{sc} = 1.25(n - 1) + 0.85$ Å. The displacement was determined from the crystal structure to be $d = 4.0$ Å.

celles because $R_C/l \leq 1.04$. This ratio corresponds to a difference of less than 1 Å between the maximum extended hydrophobic chain length and the radius of the cylindrical micelle. Therefore, these short-chain diacylphospholipids very likely form cylindrical micelles. Lipids with $R_C/l > 1.05$ form lipid bilayers. Cylindrical micelles were actually described for the short-chain PCs (Eastoe et al., 1998; Tausk et al., 1974a,b). To our knowledge, the shapes of micelles of the other lipid classes have not been reported in the literature. Tausk et al. (1974a,b) also pointed out that the micelles of diC_7PC and especially diC_8PC are very large and polydisperse, whereas those of diC_6PC have defined sizes and shapes. The values of R_C/l increase with chain length for each lipid class. The most dramatic increase is observed for the PEs. In excellent agreement with this prediction, we demonstrated by ^{31}P -NMR that lipids of this class have the shortest chain-length threshold for bilayer formation (C_6). PGs exhibit the longest chain-length threshold for bilayer formation (C_{12}). A correlation between experiment and theory shows that lipids with $R_C/l < 1.05$ form cylindrical micelles, and lipids with greater R_C/l ratios form bilayers. Thus, the structures of supramolecular self-assemblies of diacylphospholipids can be successfully predicted by packing constraints, if the displacement between the two acyl chains is taken into account.

The reason for the increased chain length threshold of PGs to form bilayers can be attributed to the larger effective headgroup surface area (66 Å²) at low salt concentration. This compared to PC (62 Å²) larger value presumably arises from electrostatic repulsion between the negatively charged headgroups. Watts et al. (1981) reported that the electro-

static repulsion between the charged headgroups in the gel-phase of $diC_{16}PG$ lead to a 30% increase of the surface area compared to the protonated form of this lipid. The volume of the nonhydrated glycerol headgroup is actually smaller than that of the choline headgroup. The effect of increasing ionic strength on the threshold of PGs to form bilayers is also easily explained with the above-described geometric packing model. For example, if we assume that increasing the NaCl concentration to 2 M will effectively eliminate the charge repulsion and yield an effective headgroup surface area smaller than that of PC (62 Å²), we predict a micelle-to-bilayer transition at the C_9 homolog. In agreement with this expectation, $diC_{10}PG$ was found to form bilayers in 2 M NaCl (Fig. 3). Similarly, diC_8PS , which forms micelles in low salt, was found to form bilayers in high salt environments.

In a previous Raman and differential scanning calorimetry study, it was concluded that $diC_{10}PC$ forms micelles above $-8.5^\circ C$ (Huang et al., 1982). These authors arrived at their conclusion based on indirect arguments. Our ^{31}P -NMR data provide direct and unequivocal evidence for a bilayer structure of $diC_{10}PC$. The Raman spectral changes that were observed by Huang et al. (1982) could be interpreted equally well with a bilayer structure in which the hydrocarbon chains are more flexible (less ordered) than in thicker, better ordered lipid bilayers. Our ^{31}P -NMR spectra show no indication of an unusual headgroup structure or order for this lipid, but the CSA of diC_9PC is substantially smaller than that of the longer-chain homologs. This is a clear indication of more headgroup motion in diC_9PC bilayers.

TABLE 2 Correlation of theoretical micelle radii of spherical and cylindrical micelles and threshold chain length for bilayer formation

| | n_c | v^* (\AA^3) | a^\dagger (\AA^2) | R_s^\ddagger (\AA) | R_c^\ddagger (\AA) | $l^§$ (\AA) | R_s/l | R_c/l |
|--------------------------|----------------------|-----------------------------|-----------------------------------|------------------------------------|------------------------------------|---------------------------|-------------|-------------|
| <i>diC_nPC</i> | 8 | 432 | 62 | 20.9 | 13.9 | 13.6 | 1.54 | 1.02 |
| | 9[¶] | 486 | 62 | 23.5 | 15.7 | 14.8 | 1.58 | 1.06 |
| | 10 | 540 | 62 | 26.1 | 17.4 | 16.1 | 1.62 | 1.08 |
| | 12 | 648 | 62 | 31.4 | 20.9 | 18.6 | 1.69 | 1.12 |
| <i>diC_nPE</i> | 6 | 324 | 52 | 18.7 | 12.5 | 11.1 | 1.68 | 1.12 |
| | 8 | 432 | 52 | 24.9 | 16.6 | 13.6 | 1.83 | 1.22 |
| | 10 | 540 | 52 | 31.2 | 20.8 | 16.1 | 1.94 | 1.29 |
| | 12 | 648 | 52 | 37.4 | 24.9 | 18.6 | 2.01 | 1.34 |
| | 14 | 756 | 52 | 43.6 | 29.1 | 21.1 | 2.07 | 1.38 |
| <i>diC_nPG</i> | 6 | 324 | 66 | 14.7 | 9.8 | 11.1 | 1.33 | 0.88 |
| | 8 | 432 | 66 | 19.6 | 13.1 | 13.6 | 1.44 | 0.96 |
| | 10 | 540 | 66 | 24.5 | 16.4 | 16.1 | 1.52 | 1.02 |
| | 12 | 648 | 66 | 29.5 | 19.6 | 18.6 | 1.58 | 1.06 |
| | 14 | 756 | 66 | 34.4 | 22.9 | 21.1 | 1.63 | 1.09 |
| <i>diC_nPS</i> | 6 | 324 | 62 | 15.7 | 10.5 | 11.1 | 1.41 | 0.94 |
| | 8 | 432 | 62 | 20.9 | 13.9 | 13.6 | 1.54 | 1.02 |
| | 10 | 540 | 62 | 26.1 | 17.4 | 16.1 | 1.62 | 1.08 |
| | 12 | 648 | 62 | 31.4 | 20.9 | 18.6 | 1.69 | 1.12 |
| | 14 | 756 | 62 | 36.6 | 24.4 | 21.1 | 1.73 | 1.16 |

*The hydrocarbon chain volume (v) of the two acyl chains was calculated according to Marsh (1990): $v_{hc} = 2 \cdot [(n_c - 2) \cdot v_{CH_2} + v_{CH_3}]$, with $v_{CH_2} = 27 \text{ \AA}^3$ and $v_{CH_3} = 54 \text{ \AA}^3$. The hydrophobic volumes are in agreement with published partial specific volumes of PC and PE (Knoll et al., 1981; Koynova and Hinz, 1990; Nagle and Wilkinson, 1978; Wiener et al., 1988).

[†]Headgroup surface areas (a) were from Cevc (1993) for PC, from Seddon et al. (1984) and Tate and Gruner (1989) for PE, from Marsh (1990) for PG, and from Mattai et al. (1989) for PS.

[‡]Radii of spherical (R_s) and cylindrical (R_c) micelles were calculated according to Eqs. 3 and 4.

[§]The maximum extended chain length of the phospholipids (l) were calculated as described in the legend to Fig. 6.

[¶]Lipids in boldface indicate the minimal threshold of the acyl chain length that is required for the formation of lipid bilayers.

The ^{31}P -NMR spectra of *diC₁₀PG* in different salt concentrations give some hints on what might happen at the micelle-to-bilayer transition. We envisage that the cylindrical micelles grow in size as the v/a ratio is effectively increased by decreasing the surface charge density. They eventually become so large that they will connect to one another and form a worm-like highly flexible network of lipid tubes. Connecting long lipid tubes would gradually hinder their rapid rotation around perpendicular axes and would result in ^{31}P -NMR spectra that are characterized by rapid rotational averaging of molecules around the tubular cylinders. This behavior is equivalent to the motional averaging of lipids in the better-known H_{II} phases. Therefore, H_{II} and tubular phase ^{31}P -NMR spectra will look similar if the headgroup structures are similar in the two phases. Spectra of these phases are characterized by powder patterns that are inverted and exhibit half the CSA of the bilayer spectra of the same lipid (Cullis and de Kruijff, 1979; Seelig, 1978). Such spectra are indeed observed superimposed on normal bilayer spectra at intermediate salt concentrations (Fig. 3). In fact, micellar, tubular, and bilayer phases appear to coexist in this transition region. As v/a is further increased, the tubes flatten out and form pure bilayer phases. The spectrum of *diC₁₀PG* also exhibits a superimposed inverted powder-pattern component at low salt

concentration, but with a much reduced CSA, indicating very efficient motional averaging. A manifestation of the formation of tubular phases may also be the appearance of the so-called ‘‘cloud point,’’ i.e., a point at which surfactant micelles begin to macroscopically phase-separate from bulk solvent (Mitchell et al., 1983; Zulauf, 1990). In agreement with these observations, Tausk et al. (1974a) have described macroscopic phase separations of micellar *diC₈PC* solutions.

Apart from being excellent detergents for the purification and reconstitution of membrane proteins (Hauser, 2000; Kessi et al., 1994) short-chain diacylphospholipids have recently found application in membrane protein crystallization and as useful environments for studies of membrane proteins by NMR spectroscopy (Sanders and Oxenoid, 2000). Particularly, *diC₆PC* has been used in recent high-resolution NMR studies of membrane proteins (Fernández et al., 2001; Marassi et al., 1999). The results on the organization of these micelles obtained in this study have implications for high-resolution structural studies of membrane proteins by NMR spectroscopy. Fernández et al. (2001) estimated from the linewidths of their spectra that the mixed *diC₆PC*/protein micelles had a molecular mass of 60 kDa, of which 16.5 kDa was protein. This micelle size is much larger than the 16 kDa reported by Tausk et al.

(1974b). However, if the lipids assemble in rod-shaped micelles as described here, their anisotropic tumbling might give rise to much broader than expected resonance lines and, therefore, could have been the reason for an overestimation of the particle size by Fernández et al. (2001). Although the asymmetric shape of short-chain diacylphospholipid micelles may present a disadvantage for obtaining spectra of the highest possible resolution of membrane proteins, this shape may be put to good use by orienting membrane proteins in high magnetic fields. Residual dipolar couplings obtained from oriented samples could potentially greatly facilitate structure determinations of membrane proteins, as has been amply demonstrated for soluble proteins (Bax et al., 2001).

CONCLUSIONS

The formation of supramolecular assemblies is dictated by the hydrophobic effect and by geometric packing constraints of the monomeric amphiphiles in the assembly. A simple theory, which has been previously developed for single-chain amphiphiles, has been shown here to be valid also for zwitterionic and charged diacylphospholipids. To explain the supramolecular structure of diacylphospholipids, the geometric packing condition must include the displacement between the two hydrocarbon chains of these lipids. The theory predicts whether a given lipid forms spherical micelles, cylindrical micelles, or bilayers. The short-chain diacylphospholipids used in this study most likely form cylindrical rather than spherical micelles. A tubular intermediate phase appears to be present at the transition from the micelle to the bilayer phase, as observed for charged phosphatidylglycerol by variation of the ionic strength.

NOTES

1. The term CMC is used for the critical concentration of phospholipids that is required for the transition of monomeric phospholipids to any supramolecular assembly, independent of geometry.

2. The spectra of *diC₁₂PC* and *diC₁₂PS* show signs of some macroscopic ordering of the bilayers in the magnetic field (Seelig et al., 1985). The reasons for this behavior of some, but not other samples is unknown.

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