Small Angle X-Ray Scattering Studies of Magnetically Oriented Lipid Bilayers

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ABSTRACT Magnetically oriented lipid/detergent bilayers are potentially useful for studies of membrane-associated molecules and complexes using x-ray scattering and nuclear magnetic resonance (NMR). To establish whether the system is a reasonable model of a phospholipid bilayer, we have studied the system using x-ray solution scattering to determine the bilayer thickness, interparticle spacing, and orientational parameters for magnetically oriented lipid bilayers. The magnetically orientable samples contain the phospholipid L-α-dilauroylphosphatidylcholine (DLPC) and the bile salt analog 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) in a 3:1 molar ratio in 70% water (w/v) and are similar to magnetically orientable samples used as NMR media for structural studies of membrane-associated molecules. A bilayer thickness of 30 Å was determined for the DLPC/CHAPSO particles, which is the same as the bilayer thickness of pure DLPC vesicles, suggesting that the CHAPSO is not greatly perturbing the lipid bilayer. These data, as well as NMR data on molecules incorporated in the oriented lipid particles, are consistent with the sample consisting of reasonably homogeneous and well dispersed lipid particles. Finally, the orientational energy of the sample suggests that the size of the cooperatively orienting unit in the samples is 2×10^7 phospholipid molecules.

INTRODUCTION

Because of their anisotropic diamagnetic susceptibilities, many biological molecules and complexes orient in a magnetic field. Magnetic orientation has been exploited in a number of cases for the study of macromolecular structure and assembly (Chabre, 1975; Torbet, 1987, 1992). Among the systems of biological interest for which magnetic orientation proves beneficial are mixtures of lipids that orient in a magnetic field (Seelig et al., 1985; Speyer et al., 1987). These provide a useful medium for studying the conformations and dynamics of membrane-associated molecules using nuclear magnetic resonance (NMR) spectroscopy (Sanders et al., 1994). A number of cell surface carbohydrates and protein/carbohydrate complexes have been studied in a membrane environment using these methods. Since the structural organization of the oriented lipid particles is of fundamental interest and is relevant to the use of the oriented media as a model for biological membranes in the NMR studies, we used small angle x-ray scattering to study the morphology of lipid samples that orient in a magnetic field.

Among the lipid mixtures that have been found to orient in a magnetic field, samples composed of a 3:1 molar ratio of the phospholipid dimyristoyl phosphatidylcholine (DMPC) and the zwitterionic bile salt analog 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) in 70% water (w/v) have been used for most of the structural work employing magnetically oriented lipid samples. The average orientations of the long axes of the acyl chains of the phospholipid molecules in these samples are approximately perpendicular to the applied magnetic field (Sanders and Prestegard, 1990). The morphology of the DMPC/CHAPSO system was studied recently by measuring anisotropic diffusion rates of water using pulsed-field gradient NMR methods (Chung and Prestegard, 1993). Modeling the diffusion of water as stochastic movement over fixed sites in a lattice, the results are consistent with the DMPC/CHAPSO medium being composed of disk-shaped particles ~51 Å thick and having a diameter of ~200 Å.

Considerably more work has been done on related mixtures of egg phosphatidylcholine (PC) with bile salts. Most of these studies have been carried out in the absence of an applied magnetic field. X-ray scattering (Müller, 1981) and quasielastic light scattering (Mazer et al., 1980) results using PC/bile salt mixtures with molar ratios near 1:1 and total amphiphile concentrations of ~10% (w/v) are consistent with disk-shaped lipid bilayers between 100 and 400 Å in diameter. Neutron scattering studies conducted on PC/bile salt mixtures in a 0.9:1 molar ratio and containing 5% total amphiphile in water (w/v) are consistent with small globular particles dominating the morphology of the samples (Hjelm et al., 1990). Other morphologies, including extended, rodlike structures, have been observed in more dilute PC/bile salt mixtures (Hjelm et al., 1990; Walter et al., 1991).

While the molecular components of the PC/bile salt systems are similar to the magnetically orientable DMPC/CHAPSO samples, the lipid/detergent ratios and the total

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concentration of amphiphiles are significantly different. Because morphology in the lipid/bile salt samples seems to be extremely sensitive to both lipid/bile salt ratios and to total concentration of amphiphile, it is unclear whether the results from the PC/bile salt samples can be extrapolated to the DMPC/CHAPSO samples that orient in magnetic fields. Further, the high magnetic fields used in NMR studies could affect the morphology of the DMPC/CHAPSO mixtures.

Small angle x-ray scattering is well suited to provide structural information on the oriented lipid particles. X-ray scattering is sensitive to the distribution of electron density within the sample and has been used to determine the transbilayer distribution of membrane components, including lipids, proteins, and water molecules (Blasie et al., 1985; Wiener and White, 1992). Comparison of the transbilayer distribution of electron density in our samples with that of a bilayer of pure lipid should reveal any large perturbation of bilayer structure caused by the presence of CHAPSO in the sample. If the lipid particles are ordered with respect to one another, the continuous diffraction pattern from the electron density profile of the lipid bilayer will exhibit additional interference effects, corresponding to the interparticle spacing in the sample. Lamellar stacking has been observed in lipid dispersions (Sömjen et al., 1991) and is of interest here, because interparticle packing may perturb the conformations of cell surface receptors observed in NMR studies. Finally, small angle x-ray scattering can be used to determine orientational parameters for the system. Since small angle x-ray scattering occurs on a much faster time scale than NMR, the information is complementary to that obtained from NMR experiments.

MATERIALS AND METHODS

Permanent magnet assembly

To orient the lipid samples in the x-ray apparatus, a permanent magnet assembly was constructed based on a published design (Oldenbourg and Phillips, 1986). The magnet assembly provides a uniform, strong magnetic field in a small volume between the tips of two conical pole pieces. The pole pieces are 2 mm in diameter at their tips. Each pole piece is attached to a neodynium-iron-boron magnet of 3/4 inch in diameter, and 3/16 inch thick (Edmund Scientific, Barrington, NJ). Each magnet is fixed to a side piece in the magnet assembly, and the two side pieces are attached to the base. The pole pieces, side pieces, and base are made from low carbon steel. The overall dimensions of the assembly are 3 inches in length, 1 inch in width, and 1¼ inches in height.

The position of the side pieces on the base is adjustable, allowing the gap between the pole pieces and thus the strength of the magnetic field to be varied. In the studies reported here, a gap of 1.0 mm was used between the pole pieces, producing a magnetic field of ~2 T. When the magnet assembly is placed in the x-ray scattering apparatus, the directions of the magnetic field, the long axis of the capillary tube containing the sample, and the x-ray beam line are mutually orthogonal.

Sample preparation

A sample with L-α-dilauroylphosphatidylcholine (DLPC) and CHAPSO in a 3:1 molar ratio in 70% distilled water (w/v) was prepared in a 5 mm NMR tube as described previously (Sanders and Prestegard, 1990). DLPC, rather than DMPC, was used in the scattering studies because an oriented nematic phase in the DLPC/CHAPSO system is found to occur near 20°C. Orientation at room temperature eliminates the need for temperature control of the permanent magnet assembly. A sample for the x-ray scattering experiments was prepared by transferring ~50 μl of the DLPC/CHAPSO mixture from the NMR tube to a 1 mm glass capillary tube (Charles Supper Company, Natick, MA), spinning it down in a hand centrifuge and sealing the top of the capillary tube with a flame.

Instrumentation

The x-ray source is a Rigaku 300 rotating anode x-ray generator operating at 50 kV and 180 mA. The x rays exit the source through a 0.5 mm × 0.5 mm slit into a monochromator adjusted to select x rays with a wavelength of 1.54 Å. After a 50 cm helium path, the x rays pass through a 0.6 mm platinum/iridium pinhole (Ted Pella, Inc., Redding, CA) and then through the sample. A helium cone with mylar windows on the front and back provides a helium path between the sample and the beamstop, which is 4 cm in front of the detector. The direct beam is absorbed by a lead beam stop 5 mm in diameter. X rays are detected with a Xuong–Hamlin multiwire area detector (Hamlin et al., 1981) positioned 1.25 m from the sample.

Data processing

The binary data files were converted to ASCII text files using the FORTRAN program DUMP (written by H. W. Wyckoff). Grayscale plots of pixel intensities were made using Mathematica (Wolfram Research, Champaign, IL). The remainder of the software used in data processing was written in C and implemented on a Sun Sparc I workstation. The position of the center of the direct beam was determined from a collected data set by finding the position on the area detector with equal intensity on either side in both the meridional and equatorial directions. The proportion of lipid particles within the sample with orientations satisfying the Ewald sphere condition for the observation of diffracted x rays decreases with increasing scattering angle. For this reason, each pixel was multiplied by a Lorentz factor of $s = 2\sin(\theta)/\lambda$, where θ is one-half the scattering angle, before sector integration. Since the total intensity at each scattering angle used in subsequent analyses is calculated by integrating over an arc on the area detector, no further correction is required.

The area detector image acquired for the DLPC/CHAPSO sample was corrected for background scatter of water by subtracting an area detector image of a water sample with the same total exposure time as for the lipid sample. The pixel intensities from the water sample were first adjusted so that total scattered intensity is zero at $s > 0.09$ Å^{-1} to account for differential absorption of the direct beam by the water and lipid samples.

Since the data were truncated for $s < 0.005$ Å^{-1}, the Patterson inversion of the scattered intensity in the direction normal to the bilayer is equivalent to the autocorrelation function of the electron-density contrast of the bilayer with the surrounding medium. Assuming centrosymmetry, the Patterson function, $P(x)$, may be computed as:

$$P(x) = \sum_i C(s)\cos(2\pi s_i)\Delta s \qquad (1)$$

Corrected intensity, denoted $C(s)$, refers to scattered intensity corrected by the Lorentz factor and for background scatter of water.

RESULTS AND DISCUSSION

Installation of the permanent magnet assembly into the x-ray apparatus allows the magnetically orientable lipid samples to be studied in a magnetic field using small angle x-ray scattering. A sample containing DLPC/CHAPSO in a 3:1 molar ratio in 70% water (w/v) was allowed to equilibrate in the permanent magnet holder for 4 h at 20°C before

a 1-h exposure was collected. The raw scattered intensities after background subtraction are shown in Fig. 1. Enhanced scattered intensity along the meridian, defined as the direction perpendicular to the magnetic field, is clearly evident in Fig. 1. Exposures were collected after up to 24 h of equilibration in the magnetic field, but no further change in sample orientation was observed. Preferential orientation of the sample was not observed when the sample was placed in the sample holder, but the magnets were removed (data not shown). Since constructive scattering for lipid bilayers only occurs in the direction normal to the bilayer (Wilkins et al., 1971), the increased intensity in the meridional direction demonstrates that the bilayers are oriented preferentially with their normals perpendicular to the applied magnetic field. This is consistent with results observed in NMR experiments and suggests that the samples are exhibiting behavior similar to that observed in the higher magnetic fields of superconducting magnets.

Lipid bilayer thickness and interparticle spacing

After application of the Lorentz correction, the intensities of the scattered x rays integrated in a wedge along the meridional and equatorial directions were plotted as a function of scattering angle. The results are shown in Fig. 2. A maximum in the scattered intensity is observed between s = 0.030 and 0.035 Å^{-1}. It is likely to correspond to the maximum in scattered intensity at $s = 1/D$, where D is the spacing between the phosphate groups across the bilayer, predicted from the continuous transform of the electron density distribution of a membrane bilayer along the bilayer normal (Wilkins et al., 1971). The scattered intensity in this region is similar to that observed for pure DLPC vesicles (Lewis and Engelman, 1983).

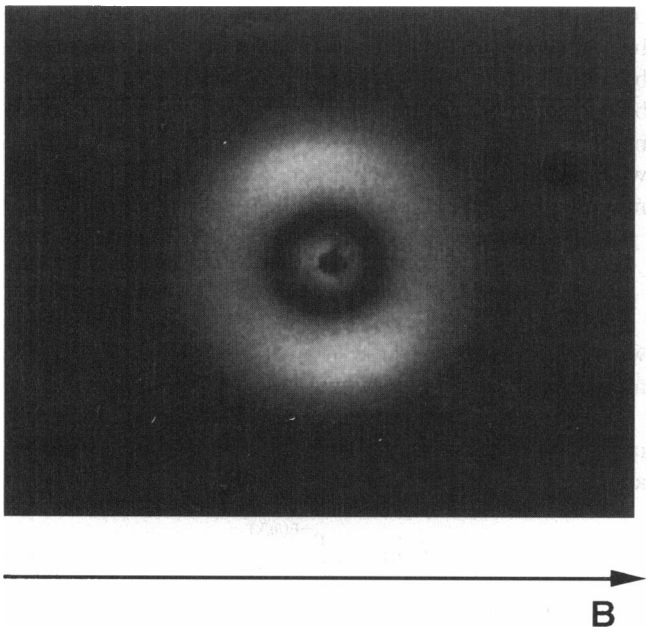

FIGURE 1 Scattered small angle x-ray intensity from a sample containing DLPC/CHAPSO in a 3:1 molar ratio in 70% water (w/v) in an applied magnetic field after background subtraction of water. The direction of the applied magnetic field is denoted by B. Data at $S < 0.005$ Å^{-1} are masked because of inaccuracies in background subtraction at very small angle. The intense scattering appears to be misaligned relative to the meridional axis of the detector, probably because of slight misalignment of the magnets in the sample holder.

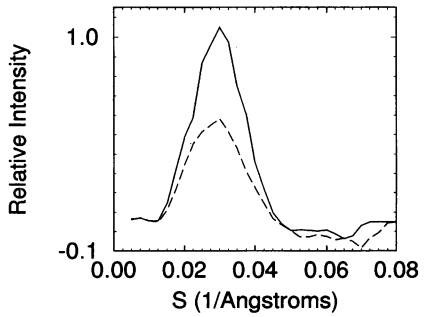

FIGURE 2 Normalized small angle x-ray scattering intensity from the DLPC/CHAPSO sample in an applied magnetic field along the meridional (——) and equatorial (- - - -) directions as a function of scattering angle. The intensities in the meridional and equatorial directions are a sum over a range of 40° about the meridional and equatorial axes, respectively.

More precise bilayer parameters can be determined from the Patterson inversion of the data shown in Fig. 2. Calculating $P(x)$ according to Eq. 1 with the index i incremented by 0.0025 Å^{-1} between s = 0.005 Å^{-1} and s = 0.1 Å^{-1} produces the Patterson function shown in Fig. 3. The positive peak at 30 Å corresponds to the thickness of the bilayer. It is close to the value of 30.5 ± 1 Å determined previously for DLPC vesicles (Lewis and Engelman, 1983). Furthermore, a titration of DLPC with CHAPSO, with molar DLPC/CHAPSO ratios ranging from 6:1 to 2:1 showed no significant variations in the thickness of the lipid bilayer (data not shown). The results suggest that the presence of CHAPSO in these samples does not substantially alter the bilayer structure of the lipid particles.

In addition to a positive peak expected in the Patterson function at the distance between the phosphate headgroups, additional positive peaks at longer distances may indicate regular spacing of the lipid particles in the sample. The absence of these peaks in the Patterson functions shown in

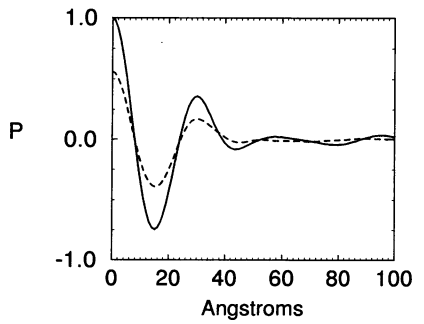

FIGURE 3 The normalized Patterson function calculated for the data shown in Fig. 2 using Eq. 1.

Fig. 3 indicates that the sample is likely to be composed of dispersed lipid particles rather than multilayers. This result is encouraging for NMR studies using the lipid medium, because it indicates that the conformation and dynamics of molecules associated with the oriented bilayers are not likely to be affected significantly by interactions with neighboring lipid particles.

Although the incorporation of CHAPSO in the interior of the lipid bilayer may not cause detectable changes in the bilayer thickness, the observation that the DLPC bilayer is not grossly deformed by the addition of CHAPSO is certainly consistent with a model in which the particles in the DLPC/CHAPSO samples are disk-shaped micelles composed primarily of DLPC with the hydrophobic face of CHAPSO stabilizing the exposed acyl chains on the edge of the micelle. A test of the reasonableness of this model is whether the exposed hydrophobic area on the edge of the disk is sufficient to accommodate all of the CHAPSO molecules. In DLPC vesicles in solution, the measured surface area of each DLPC molecule is 67 Å^2, and the hydrophobic dimension of each DLPC bilayer is 20 Å (Lewis and Engelman, 1983). If all of the CHAPSO molecules are accommodated around the edge of the disk-shaped micelle and the micelles are 200 Å in diameter (Chung and Prestegard, 1993), a hydrophobic surface area of 40 Å^2 would be allowed for each CHAPSO molecule. The hydrophobic face of CHAPSO consists of four fused, cyclic hydrocarbons. The maximal area of this portion of CHAPSO projected onto a plane is 18 Å^2. The CHAPSO in the sample could therefore be easily accommodated around a DLPC disk 200 Å in diameter, and indeed the calculation suggests that a slightly larger disk may be stabilized even more effectively by interaction of a larger fraction of its exposed hydrophobic surface with the detergent molecules.

Orientational parameters

X-ray scattering occurs on a time scale that is much faster than the time scale of the motions of the lipid particles, so the intensity observed from the scattering of x rays by the particles is distributed in a circle on the area detector, reflecting the instantaneous orientational disorder in the sample. The length of the scattering vector, s, and an angle α, defined as the polar angle relative to the applied magnetic field, are convenient coordinates to denote any position on the area detector. Because the scattering appears to be dominated by the electron density profile of the bilayer, the relative intensity of scattered x rays at any angle α is proportional to the number of particles in the sample with bilayer normals oriented to give diffracted intensity in that direction. The filled circles in Fig. 4 show the normalized angular distribution of x-ray scattering intensities between $s = 0.020$ and $s = 0.040$ Å^{-1} for the DLPC/CHAPSO sample from the data shown in Fig. 1. The total scattered intensity in a range of s values near the maximum at $s = 0.030$ Å^{-1} was chosen to enhance the statistical accuracy of the determined angular distribution.

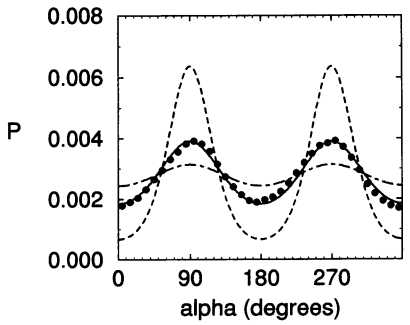

FIGURE 4 The normalized angular distribution of scattered x-ray intensity between $S = 0.020$ and $S = 0.040$ Å^{-1}. The polar angle, α, is defined as 90° at the position on the top half of the area detector with maximum scattered intensity and positive angles are in the counterclockwise direction. The graph includes the experimental data shown in Fig. 1 (●) and calculated intensity distribution using Eqs. 2 and 3 with $N = 6 \times 10^7$ lipid molecules (- - - -), $N = 2 \times 10^7$ lipid molecules (–·–·–) and $N = 0.6 \times 10^7$ lipid molecules (–●–●–).

The main angular dependent term in the free energy of the lipid particles is due to the interaction of the applied magnetic field with the lipid molecules. The tendency for phospholipids to orient in magnetic fields is attributed to the diamagnetic anisotropy, $\Delta\chi$, of the hydrocarbon chains. A value in cm-g-s system units of $\Delta\chi = -0.96\ (\pm 0.1) \times 10^{-8}$ erg cm^{-3} G^{-2} was measured for DMPC in L_α phase bilayers (Scholz et al., 1984). Since DLPC only differs from DMPC by having two fewer carbons on each acyl chain, the diamagnetic anisotropies of DLPC and DMPC are likely to be quite similar. Defining $\Delta E(\theta)$ as the difference in energy between an array of lipid molecules in bilayers with the bilayer normals oriented at an angle θ relative to the applied magnetic field and an array of lipid molecules in bilayers with no preferential orientation, the orientational energy may be written as

$$\Delta E(\theta) = \frac{1}{3} N \Delta\chi B^2 \left(\frac{3\cos^2\theta - 1}{2} \right) \qquad (2)$$

where B is the magnetic field and N is the number of lipid molecules in the oriented unit.

The angular distribution of particles in a sample, $P(\theta)$, may be determined using the energies calculated in Eq. 2 and the Boltzmann equation, resulting in

$$P(\theta) = \frac{e^{-E(\theta)/kT}}{\sum_\theta e^{-E(\theta)/kT}}. \qquad (3)$$

where k is Boltzmann's constant and T is the temperature. From comparison of the calculated angular distribution of particles for various values of N with the angular distribution of scattered, small angle x-ray intensity from the DLPC/CHAPSO sample, a value for N can be estimated. The lines in Fig. 4 show calculated angular distributions for three different values of N, assuming the lipids have anisotropies in susceptibilities similar to those of extended

bilayers. The distribution corresponding to the value $N = 2 \times 10^7$ lipid molecules fits the data quite well.

The angular distribution of scattered x rays provides insight into the homogeneity of the x-ray scattering sample and into the size of the domain within the sample that is orienting as a cooperative unit. Since the observed angular distribution of particles fits the calculated distribution using a single value of N, the x-ray scattering data are consistent with the sample consisting of a homogeneous set of oriented lipid particles. However, a more complicated model in which heterogeneous particles have an average size of 2×10^7 lipid molecules cannot be ruled out on the basis of the x-ray scattering data alone. NMR studies of the orientation and dynamics of the lipid particles provides further evidence that the samples are indeed quite homogeneous (Sanders et al., 1994). The observation of only single sets of anisotropic chemical shifts for the ^{31}P in the headgroup of DMPC and the ^{13}C carbonyls in DMPC and CHAPSO demonstrates that the lipid particles are both oriented and reasonably homogeneous in the high magnetic fields of superconducting magnets and suggests that they are likely to be homogeneous in the lower fields used in the x-ray scattering studies as well.

The observation of an oriented unit consisting of 2×10^7 lipid molecules could be explained either by a single particle of this size or by the correlated motion of a number of smaller particles. Cooperative orientation of the lipid particles is apparent from observations in NMR studies that orientation only occurs in samples containing at least 5 wt % lipid (Sanders et al., 1994). Steric interactions among lipid particles in the samples may provide sufficient energy for cooperative orientation of the lipid particles at these higher lipid concentrations. Other effects, such as electrostatic or magnetic interactions, on the orientational properties of molecules in the sample have not yet been demonstrated (B. J. Hare and J. H. Prestegard, unpublished data).

The size of the cooperatively orienting unit may be inferred from the number of lipid molecules it contains. If the lipid particles are disk shaped with a diameter of 200 Å (Chung and Prestegard, 1993) and each lipid molecule has an area of 67 $Å^2$ (Lewis and Engelman, 1983), 2×10^7 lipid molecules could be accommodated in ~20,000 disks. This cooperatively orienting domain would occupy 0.06 μm^3 in a sample composed of 30% lipid by weight. These results imply that the interparticle interactions that cause the sample to orient at the low fields used here extend over only a small fraction of the total sample volume. Shorter range interparticle correlations among lipid particles within the cooperative unit were not observed in the Patterson function in Fig. 3, suggesting that even neighboring particles are substantially disordered relative to one another in the sample. This is likely due to some independent wobbling of each lipid particle within the oriented domain.

CONCLUSIONS

Orientation of a DLPC/CHAPSO sample in a 2 T field was demonstrated, allowing determination of the bilayer thickness, interparticle spacing, and orientational parameters for the sample. Sample orientation in magnetic fields produced by permanent magnets is advantageous, because it makes possible biophysical characterization of sample orientation and overall morphology as well as the study of the structures of molecules that associate with the lipid bilayers. Observation of x-ray or neutron scattering at smaller angles along the equatorial axis of the detector may allow direct determination of particle size and shape and the distribution of CHAPSO in the bilayers. Small angle x-ray scattering from samples that contain membrane-associated proteins may also provide structural information that is complementary to the information provided by NMR spectroscopy.

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