Direct spectroscopic observation of inner and outer hydrocarbon chains of lipid bilayer vesicles

(Kenneth J. Longmuir and F. W. Dahlquist)

ABSTRACT Distearoylphosphatidylcholines labeled with gem-difluoro groups at selected positions of the fatty acid chains have been prepared. These lipids were incorporated into sonicated vesicles and were examined by fluorine nuclear magnetic resonance spectroscopy. All spectra are characterized by two well-separated fluorine resonances. Paramagnetic ions added to the exterior solution of vesicles of fluorinated lipid broaden the upfield resonances, whereas ions trapped in the vesicle interior broaden the downfield resonances. This demonstrates that the fluorine chemical shift of these compounds is sensitive to differences between the inner and outer monolayers of sonicated vesicles.

Considerable attention has been given recently to the nuclear magnetic resonance (NMR) behavior of hydrocarbon chains in lipid bilayers (1-3). The nuclear magnetic relaxation data and the residual observed quadrupole splitting data so obtained have been used to determine the average mobility and anisotropy of motion at various positions along the chain.

Many of these NMR studies have involved sonicated preparations of phosphatidylcholine (PC), which result in solutions of single bilayer vesicles of relatively small diameter. These spherical shells of lipid have such a high degree of curvature that a significant excess of PC populates the outer monolayer of the bilayer compared to the inner one (4). Some comment has been directed toward the structural differences between the inner and outer monolayers, particularly with regard to the head group (5), but spectroscopic resolution of these two classes of hydrocarbon chains in the bilayer interior has not been achieved.

This communication describes the synthesis and fluorine magnetic resonance properties of distearoyl PC labeled with gem-difluoro groups at selected positions along the fatty acid chains. Such efforts were undertaken because of the sensitivity of the fluorine chemical shift to changes in the microscopic environment surrounding the fluorine nucleus. With these specifically labeled compounds, we have made several interesting observations concerning the structure of lipid bilayers. Here, we show that the fluorine NMR technique resolves the inner and outer monolayers of the hydrocarbon interior of sonicated lipid bilayer vesicles.

MATERIALS AND METHODS

Materials. 12-Hydroxystearic acid and L-α-glycero-phosphorylcholine were obtained from ICN Pharmaceuticals, Fluoreze-M from CR, and stearic acid from Sigma. Dicyclohexylcarbodiimide and carbonyldimidazole were obtained from Aldrich. Phospholipase A and dipalmitoyl PC were supplied by Calbiochem. All solvents were analytical grade.

Synthesis of 1,2-bis(12',12'-difluorostearoyl)-sn-glycero-3-phosphorylcholine [bis(12',12'-F2)-PC] and 1,2-bis(7',7'-difluorostearoyl)-sn-glycero-3-phosphorylcholine [bis(7',7'-F2)-PC]. Established procedures were used to synthesize 12-ketomethylstearate (6) and 7-ketomethylstearate (7). These materials were fluorinated with 1:1 mixtures of 0.8 M MoF6 (Fluoreze-M) and 0.8 M ketone in CH2Cl2 according to Mathey and Bensoan (8). The fluorinated methyl esters were purified and saponified to the free acid. L-α-Glycero-phosphorylcholine and 12,12-difluorostearic acid were coupled by the acid anhydride procedure of Robles and van den Berg (9) to give bis(12',12'-F2)-PC (structure I). L-α-Glycero-phosphorylcholine and 7,7-difluorostearic acid were coupled via the acyl imidazole procedure of Boss et al. (10) to give bis(7',7'-F2)-PC (structure II).

CH3(CH2)m-CF2j(CH2)n-CO-CH2
CH3(CH2)m-CF2j(CH2)n-CO-CH2

H2C=O-P-CH2CH2N(CH3)3

Abbreviations: NMR, nuclear magnetic resonance; PC, phosphatidylcholine; bis(12',12'-F2)-PC, 1,2-bis(12',12'-difluorostearoyl)-sn-glycero-3-phosphorylcholine; 1-monos(12',12'-F2)-PC, 1-(12',12'-difluorostearoyl)-2-stearoyl-sn-glycero-3-phosphorylcholine; bis(7',7'-F2)-PC, 1,2-bis(7',7'-difluorostearoyl)-sn-glycero-3-phosphorylcholine; ppm, parts per million.

Synthesis of 1-(12',12'-difluorostearoyl)-2-stearoyl-sn-glycero-3-phosphorylcholine (1-monos(12',12'-F2)-PC). Bis(12',12'-F2)-PC was hydrolyzed to the 1-lyso compound with phospholipase A (11) and purified by gel filtration. Recylation of the lyso PC with stearic acid was accomplished via the acyl imidazole procedure. The specificity of this synthesis was such that only 10-20% of the acyl chains migrated during recylation.

NMR Experiments. All lipids used in these studies were chromatographed on LH-20 Sephadex (95% ethanol solvent) at 37°. These purified materials exhibit a single spot on thin-layer chromatography.

Phospholipid vesicles were prepared by dissolving the lipid mixture in ethanol, blowing off the solvent with N2, then drying in vacuo. The lipid was suspended in 10 mM Tris-HCl buffer, pH 7.4, containing 10% D2O, and sonicated to near optical clarity (for approximately 10 min) with a Branson probe sonicator. The samples were always kept above their fluid-solid transition temperature during sonication and during subsequent NMR experiments.

NMR measurements were made with a Varian XL-100-15 spectrometer operating with a fluorine observe frequency of 94.1 MHz. All spectra were acquired in a Fourier transform mode with a Varian 620/1 computer.
All spectra were proton-decoupled. Temperatures were rounded to the nearest degree.

**RESULTS**

**19F NMR of 12',12'-difluorinated lipid in vesicles**

Fig. 1A shows the fluorine spectrum of bis(12',12'-F₂)-PC in sonicated vesicles at 69°, well above the fluid-solid transition temperature of 58°. This spectrum indicates the presence of two well-resolved fluorine resonances with apparent line widths of approximately 20 Hz each, and with a peak-to-peak separation of 19 Hz [0.20 parts per million (ppm)]. This result suggests that these nuclei exist in at least two environments within the hydrocarbon interior of the lipid bilayer, even though the fluorines label the same carbon position of the fatty acid chain.

Fig. 1B displays the fluorine spectrum of PC vesicles at 69°, where the fluorinated stearic acid is present only at the 1-position of the molecule, the 2-position being occupied by unlabelled stearic acid. Again two resolved absorption lines are observed separated by 26 Hz (0.28 ppm). Although there are small differences between this and the spectrum of bis(12',12'-F₂)-PC (see Discussion), the overall result points to at least two different environments within the bilayer. Since the labeled fatty acid occupies only the 1-position of the phospholipid, the two fluorine absorption lines are not due to differences between the two chains of the same molecule.

**19F NMR of 7',7'-difluorinated lipid in vesicles**

To explore the nature of these two hydrocarbon environments, paramagnetic ions were used to perturb the NMR spectrum of the fluorines. Numerous studies have shown that Mn(II) acts to enhance the magnetic relaxation behavior of nearby nuclei, resulting in broadening of resonances of these nuclei (12). Since this line broadening effect is inversely dependent upon the sixth power of the distance between the ion and the observed nucleus, conditions can be found such that nuclei in close proximity to Mn(II) display increased line widths, whereas those farther away remain virtually unaffected. In experiments of this kind, vesicles of bis(7',7'-F₂)-PC were useful because the fluorines are closer to their respective surfaces of the bilayer than they are to the fluorines of the other monolayer. As a result, it should be possible to selectively broaden the resonances corresponding to the outer monolayer by adding Mn(II) to the bulk solution. Similarly, the resonances derived from the inner monolayer should be selectively broadened by incorporating Mn(II) into the solution enclosed by the vesicle.

**DISCUSSION**

The work described here is concerned with the development of sensitive, nonperturbing molecular probes for investigating the hydrocarbon interior of lipid bilayers. We have approached this problem by incorporating gem-difluoro groups into the 12' and the 7' positions of the hydrocarbon chains of distearoyl PC (structures I and II, respectively). In hydrocarbon systems, the replacement of a proton by a fluorine atom represents a very minor perturbation of structure. The steric effects of the fluorine replacement are quite small, amounting to a few tenths of a kcal/mol in differential energy of interaction (13). In addition, the electrons about the fluorine are rather tightly held, are unavailable for hydrogen bonding, and are unreactive. In our hands, the fluorinated derivatives are essentially indistinguishable from their protonated counterparts in their chemical behavior.

The 19F NMR spectra of these compounds in sonicated vesicles reflect the existence of at least two different hydrocarbon environments, which are due to differences between the inner and outer monolayers of the interior of the bilayer. The selective broadening effects observed by addition of Mn(II) to the vesicle solutions enable us to make this spectral assignment, in an unambiguous fashion, with those fluorines in the inner monolayer resonating at a lower field than those contained in the outer monolayer.
As noted in the Results section, the spectrum of the lipid labeled on only one chain [1-mono-(12',12'-F₂)-PC] differs slightly from the doubly labeled bis(12',12'-F₂)-PC. We have observed the fluorine resonances of the complementary singly labeled lipid [1-stearoyl-2-(12',12'-difluorostearoyl)-sn-glycero-3-phosphorylcholine]. Under similar conditions, the resonance separation seen with this material is several hertz less than with the doubly labeled compound. This suggests that each resonance we observe with bis(12',12'-F₂)-PC is a composite of two slightly shifted contributions from the two fluorinated chains on the same lipid molecule.

In the study of bis(7',7'-F₂)-PC in sonicated vesicles, the fluorinated lipid was combined with a 5-fold excess of dipalmitoyl PC. In this system, the separation of the resonances was twice that of vesicles composed of pure bis(12',12'-F₂)-PC. The increased resolution is not due to differences between the 7' and 12' positions. Instead, the separation between absorption lines appears to depend upon the temperature of the sample, upon the size of the vesicles, and, in this case, upon the presence of a lipid of shorter chain length. In one-component systems of fluorinated lipid, the major spectral difference between the 7' and 12' positions is an increase in line width at the 7' position, but there is little increase in separation.

Gem-difluoro compounds have also been studied by Roberts and coworkers (14-16). Their work with specifically fluorinated, cyclic hydrocarbons indicates that there are large chemical shift differences among fluorine resonances corresponding to the various possible rotational isomers. For example, \( \nu_{equatorial} \approx \nu_{axial} \) for 1,1-difluorocyclohexane is 10.2 ppm (16) or about 960 Hz at 23 K (2.3 tesla). It is reasonable to expect similar magnitudes of fluorine chemical shift difference when the fluorinated carbon atom is involved in a gauche bond orientation as opposed to a trans bond orientation. In systems of sonicated vesicles, the rate of gauche–trans interconversion is rapid and should give a single, time-averaged resonance whose chemical shift depends upon the relative populations of gauche and trans configurations at the labeled center. Thus we suggest that the fluorine nuclei of the inner and outer monolayers have different chemical shifts because they have dissimilar distributions of hydrocarbon chain configurations. These conformational differences would arise from packing differences between the two monolayers. This packing difference is caused by the higher degree of curvature of the inner monolayer relative to the outer monolayer.

The spectroscopic resolution of the vesicle monolayers means that a number of interesting questions can be investigated. For example, the ratio of labeled lipid of the inner layer to the outer layer may be determined. For a vesicle composed entirely of the fluorinated derivative, this ratio is essentially a determination of the relative surface areas of the two sides of the vesicle—a measurement from which vesicle size may be deduced. For binary lipid mixtures, this ratio measures the partitioning of labeled lipid between the inner and outer layers as a function of the amount and kind of unlabeled lipid added. Finally, we
can directly examine the differential effects of temperature and of protein incorporation with respect to the two monolayers.

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