On the coordination of La^{3+} by phosphatidylserine

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ABSTRACT In a recent study by Bentz, J., D. Alford, J. Cohen, and N. Düzgünes (1988. *Biophys. J.* 53:593–607), La^{3+} was found to be more effective than Ca^{2+} in causing nonleaky fusion of phosphatidylserine vesicles. It was proposed that this difference in fusion efficiency may be due, in part, to a difference in coordination of the two cations. That is, Ca^{2+} was presumed to bind to the lipid phosphate, whereas La^{3+} was proposed to be coordinated by the serine carboxylate and amine. ^{31}P and ^{13}C NMR results presented here demonstrate that the lanthanides, Tb^{3+} and La^{3+}, are coordinated by the phosphodiester and carboxylate moieties of phosphatidylserine. Tb^{3+}-Phosphatidylserine optical experiments suggest that the serine amine does not coordinate the lanthanide below pH 10, at least not while the membrane has a net negative surface charge. Although these observations disagree with the structural details proposed by Bentz et al. (1988), they are not in conflict with their general fusion mechanism.

The work presented here also demonstrates that La^{3+} affects the inner surface phosphodiesters differently than those on the outer surface of phosphatidylserine vesicles. The vesicles studied are of an intermediate size, having diameters on the order of 150–200 nm. The cation appears to have a more immediate effect on the packing of the crowded headgroups on the inner surface. Higher levels of bound La^{3+} on the outer surface may be required to induce the same changes in headgroup conformation.

INTRODUCTION

Over the past two decades, Ca^{2+} and several other polyvalent cations have been shown to induce fusion of artifical membranes containing anionic phospholipids (cf Bentz et al., 1988). Under some conditions the fusion events have been found to preserve the integrity of the membranes as barriers, i.e., they are "nonleaky". These conditions are of particular interest because it is believed that natural membrane fusion events, such as secretion, are also nonleaky.

In one of the molecular mechanisms that has been proposed for nonleaky fusion, the cation interacts with lipid headgroups from both membranes (cf Bentz et al., 1988). This intermembrane bridge aids in disrupting the membrane structure in the regions of contact. A recent study demonstrated that the trivalent cation, La^{3+}, is much more effective than Ca^{2+} in inducing nonleaky fusion of phosphatidylserine (PS) vesicles (Bentz et al., 1988). This quality of La^{3+} was attributed to coordination with the carboxylate and amine of the serine headgroup rather than with the phosphodiester moiety, which is the primary site of attachment for Ca^{2+}. Presumably, this La^{3+} complex would be more exposed for intermembrane bridging and, therefore, more effective at preventing leakage.

These structural statements are very difficult to test for either Ca^{2+} or La^{3+} because they have no convenient spectroscopic properties which could be exploited in determining their coordination. For this reason, the paramagnetic and luminescent lanthanide, Tb^{3+}, was introduced in these studies to provide additional information about at least the lanthanide-phosphatidyl-serine complex.

MATERIALS AND METHODS

Bovine brain phosphatidylserine was used by Bentz et al. (1988) for their fusion studies, however, we have had problems with Tb^{3+} luminescence measurements using natural lipids because of low levels of fluorescent impurities and lipid oxidation. To avoid these complications, synthetic *sn*-3-dimyristoylphosphatidylserine (DMPS) was used in the work presented here. This lipid was purchased from Avanti Polar Lipids, Birmingham, AL, and used as received. Samples for the NMR experiments were prepared by suspending 40 mg of dry DMPS in 1.5 ml of 0.1 M NaCl using 99.8% D_2O as the solvent. This suspension was sonicated in a 300-W bath sonicator at 45°C until there was no further improvement in the solution clarity, i.e., 2–3 h. The pH of the DMPS solution was measured to be 5.5 using a glass electrode; no correction was made for the fact that the solvent was D_2O. The concentrations of $TbCl_3$ and $LaCl_3$ stock solutions were 79 and 87 mM, respectively. The lanthanide solutions were also at pH 5.5 and were added to the DMPS solution during the sonication period.

Both ^{13}C and ^{31}P NMR experiments were performed on a model QE-300 instrument (General Electric Co., Wilmington, MA) using a 10-mm broadband probe operating at 40.0 ± 0.1°C. At this temperature and pH DMPS is in the liquid crystalline state (Cevc et al., 1981). One pulse cycle of the experiments involved a 3-s delay with the ^1H decoupler

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off, a 3-s delay with the decoupler on to provide a nuclear Overhauser enhancement, and the decoupler on during the acquisition period after a 90°C observation pulse. A 10-mm coaxial tube assembly was used with a 0.1 mm film of a 20% trimethylphosphate/D_2O solution as a chemical shift reference. Spectra were collected with the field locked and the sample spinning at 13–20 Hz.

Tb^{3+} excitation spectra were collected on a spectrometer recently constructed for that purpose. It consists of a 1,000-W Xe arc lamp, with a half-meter prism monochromator for selection of the excitation wavelength, a cooled photomultiplier tube (−20°C) at a right angle to the excitation beam, and an interference filter for selection of the 545-nm emission band of Tb^{3+}. A model 427 current amplifier (Keithley Instruments, Inc., Cleveland, OH) was used to convert the photocurrent to a signal close to 1 V, which was digitized for computer processing using a 12-bit converter operating at 1 kHz. 500 conversions were averaged for each point in the spectra presented. The sample cell holder is jacketted and was held at 50°C, which is above the gel-liquid crystal phase transition temperature over the entire pH range studied (Cevc et al., 1981). The temperature at the cell was measured with a digital thermometer.

RESULTS

^{31}P NMR experiments

The effects of Tb^{3+} and La^{3+} on the ^{31}P NMR spectrum of DMPS vesicles are shown in Figs. 1 and 2. Tb^{3+} was chosen for comparison with La^{3+} because it is a paramagnetic lanthanide with very large contact and dipolar shift coefficients of opposite signs. Tb^{3+} binding to the phosphodiester groups in phosphatidylcholine membranes (Grasdalen et al., 1979) and lysophosphatidylcholine micelles (Halladay, 1988) induces a ^{31}P shift of roughly −300 ppm, which is dominated by the contact contribution. Likewise, Tb^{3+} induces a large negative shift in the DMPS ^{31}P resonance (Fig. 2) indicating a strong contact interaction, which is clearly evidence of direct coordination by the phosphate.

The Tb^{3+}-DMPS complex appears to be undergoing chemical exchange on an intermediate timescale, making it difficult to quantitatively determine the true chemical shift change. This statement is based on observations made with Tb^{3+}-phosphatidylcholine, which exhibits fast chemical exchange (Grasdalen, 1977; Halladay, 1988), and Tb^{3+}-phosphatidic acid, which exhibits slow chemical exchange until the negative surface charge is neutralized (unpublished results). A 1:16 Tb^{3+}-phospholipid, the line broadening induced in the phosphatidylcholine resonance is an order of magnitude less than that observed for the DMPS complex. The same ratio for the phosphatidic acid complex yields almost no line broadening and a small positive change in chemical shift. This is because it is possible to observe only the free lipid which experiences a nearest-neighbor dipolar shift and no induced relaxation. The ^{31}P resonance for the complexed phosphatidic acid is broadened too much to be observed with the instrument used. The Tb^{3+}-DMPS ^{31}P resonance is not only broadened too much for either fast or slow exchange, it is also asymmetric or skewed toward negative chemical shift, indicating intermediate exchange with a strong contact interaction for the bound state.

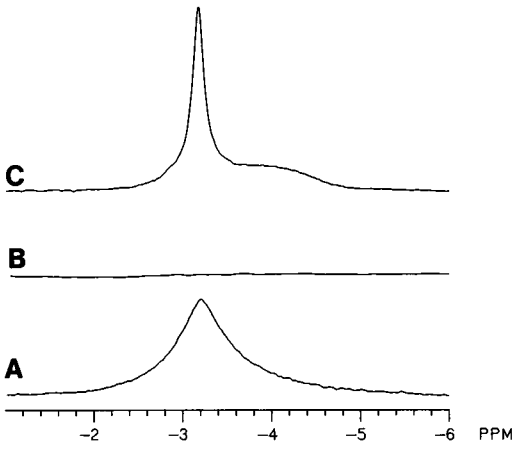

FIGURE 1 Phosphorous-31 NMR spectra of dimyristoylphosphatidylserine vesicles with and without bound lanthanide. (*A*) 41 mM DMPS in 0.1 M NaCl at pH 5.5 and 40°C with D_2O as solvent. (*B*) the same solution with Tb^{3+} at a final concentration of 2.6 mM. This sample is referred to in the text as 1:16 Tb^{3+}:DMPS. (*C*) 37 mM DMPS with 2.3 mM La^{3+} in 0.1 M NaCl at pH 5.5 and 40°C with D_2O as solvent. This sample is refered to in the text as 1:16 La^{3+}:DMPS. All chemical shifts are relative to trimethylphosphate in D_2O and each spectrum represents coaddition of 720 or more free induction decays. Apodization with an exponential function was used to reduce the noise level but resulted in 3 Hz line broadening.

The effect of diamagnetic La^{3+} on the lipid spectrum is more subtle, as shown in Fig. 1. At 1:16 La^{3+}:DMPS the ^{31}P resonance splits into two bands: a narrow band which

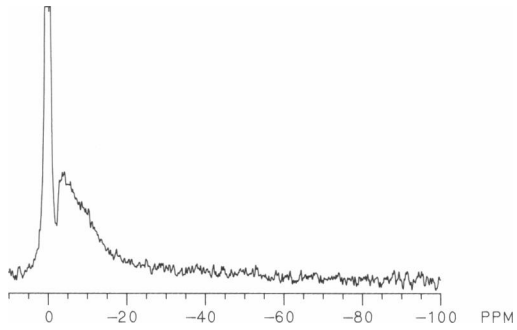

FIGURE 2 An expanded view of the 1:16 Tb^{3+}:DMPS phosphorous-31 spectrum. This spectrum is an expansion of that in Fig. 1 *B*, which was collected with a full spectral width of 100 kHz, i.e., 823 ppm at 121.5 MHz. The truncated resonance at 0 ppm is the trimethylphosphate reference and the broad, skewed band extending out to about −50 ppm is for the Tb^{3+}-DMPS complex in intermediate exchange. No other resonances were observed for up to 630 ppm from the reference.

has not moved from that of DMPS alone, and a broader band which is shifted by −0.7 ppm. Closer inspection of the band for DMPS alone (Fig. 1) reveals that it is not a single Lorentzian peak but can be fit very well with two Lorentzians, one with a width of 36 Hz and the other 132 Hz. The two bands are nearly coincident. Table 1 contains the results from regression with two Lorentzian functions for La^{3+}:DMPS ratios of 1:16, 1:8, 1:4, and DMPS alone. The area of the narrow resonance is consistently greater than that of the broad resonance by 15–30% and the two bands clearly reflect the outer (narrow) and inner (broad) leaflets of the bilayer. Table 1 also contains estimates of the vesicle diameters from the resonance areas, assuming the area per lipid molecule is equal on both surfaces and that the membrane is 5 nm thick. These vesicle diameters are closer to those of large unilamellar vesicles (200 nm) than the small unilamellar species (30 nm) usually formed by sonication.

Both bands become significantly narrower with bound La^{3+} even though the vesicles are larger than for DMPS alone. It is possible that cation binding disrupts interheadgroups interactions, such as hydrogen bonding or ion pairing, which otherwise restrict the rotational freedom of individual DMPS molecules. The apparent invariance in chemical shift for the narrow band may be mistaken as evidence that the lanthanide does not bind to the phosphates on the outer surface. This is not the case because Tb^{3+} binds to the phosphate on both the inner and outer surfaces of the vesicles, as evidenced by broadening of both ^{31}P bands.

Binding of diamagnetic cations, such as La^{3+}, is not expected to induce a significant change in the lipid ^{31}P resonance because they have very little effect on the local magnetic environment (Grasdalen et al., 1977). Therefore, the La^{3+}-induced shift of the inner phosphodiester resonance is most likely a consequence of a conformational change in the phosphodiester group which is apparently more facile for the headgroups on the inner surface. The small negative shift of the outer surface resonance at 1:4 La^{3+}:DMPS (Table 1) may be an indication that higher levels of bound La^{3+} are required to induce the conformational change on the outer surface. It is very likely that the phosphodiester conformational change is the same as that observed by Casal et al. (1987) for Ca^{2+} binding to DMPS, namely, a transition from a gauche–gauche conformation to an antiplanar–antiplanar conformation for the two ester groups. Given that La^{3+} binding to the outer surface of DMPS vesicles does not induce the conformational change until the negative surface charge is nearly neutralized, i.e., 1:4 La^{3+}:$DMPS^{1-}$ (Table 1), it seems likely that the phosphatidylserine headgroup rearrangement is electrostatically driven. Phosphatidylcholine provides a model for such an electrostatically driven conformational change. In absence of bound cation, the choline quaternary amine stays close to membrane surface because of electrostatic attraction to the layer of phosphates. As net positive charge is deposited on the surface by bound cations, the quaternary amine is forced away from the phosphates (McIntosh, 1980; Seelig et al., 1987). It is possible that the serine ammonium group is similarly held close to the layer of phosphates so long as there is a net negative charge on the membrane. Cation binding may drive the ammonium group away from the surface. The transition is probably more facile on the inner surface because of tighter headgroup packing giving rise to a greater steric rejection of the ammonium group from the surface.

TABLE 1 Parameters from nonlinear regression of DMPS and La^{3+}-DMPS ^{31}P resonances

La^{3+}:DMPS	Chemical shift*	Line width	Area (outer)‡ / Area (inner)	Vesicle§ diameter
	ppm	*Hz*		*nm*
0	−3.20	36	1.28	126
	−3.32	132		
1:16	−3.20	10	1.15	222
	−3.94	74		
1:8	−3.20	11	1.17	196
	−4.10	50		
1:4	−3.25	12	1.22	144
	−4.18	55		

*Chemical shifts are relative to the external standard: 20% trimethylphosphate in deuterium oxide. ‡Ratio of the two Lorentzian band areas obtained from the regression parameters. §Vesicle diameters estimated from the ratio of the two resonances, assuming the same area per lipid for both surfaces and a membrane thickness of 5 nm.

^{13}C NMR experiments

Natural abundance ^{13}C spectra were obtained for 1:16 La^{3+}:DMPS, Tb^{3+}:DMPS, and DMPS alone (Fig. 3). It is clear that Tb^{3+} obliterates the carboxylate resonance at 172 ppm. The two ester carbonyls are at 174 ppm. This observation is consistent with coordination of the lanthanide by the serine carboxylate (Bentz et al., 1988). The effect of La^{3+} on the carboxylate resonance is more ambiguous. The low signal level for the carboxylate resonance makes it difficult to determine whether there are two different resonances, as was observed in the ^{31}P spectra. Several of the other ^{13}C peaks are narrower in the presence of La^{3+}, which would be consistent with the ^{31}P data.

Tb^{3+}-DMPS optical experiments

Tb^{3+} is luminescent as well as paramagnetic and its excitation spectrum in phospholipid complexes has been shown to be particularly sensitive to changes in coordina-

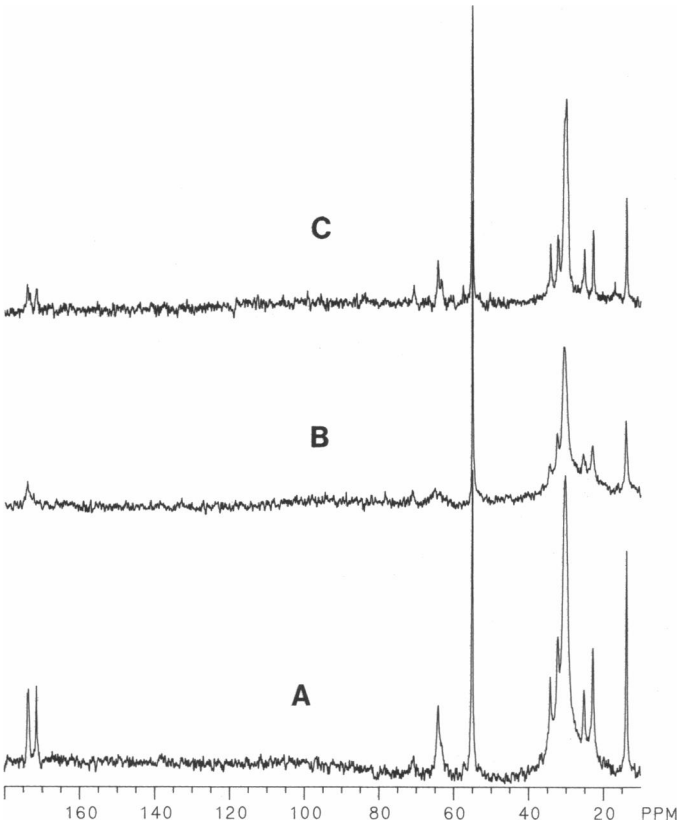

FIGURE 3 Natural abundance carbon-13 NMR spectra of dimyristoylphosphatidylserine vesicles with and without bound lanthanide. *A*, *B*, and *C* were obtained from the same samples used for the spectra in Fig. 1, *A–C*, respectively. Each spectrum was obtained using a spectral width of 20 kHz (265 ppm at 75.5 MHz) and coaddition of 10,000 or more free induction decays. Apodization was performed resulting in a 10-Hz line broadening and the external trimethylphosphate was defined as being at 55 ppm in each spectrum. The aliphatic carbon resonances for the acyl chain fall between 14 (terminal methyl) and 35 ppm; the glycerol and serine alpha and beta carbons fall between 55 and 75 ppm with one obscured by the trimethylphosphate, the serine carboxylate is at 172 ppm, and the two acyl chain carbonyls are near 174 ppm.

tion (Halladay and Petersheim, 1988). Fig. 4 represents a set of optical experiments intended to test the hypothesis put forth by Bentz et al. (1988) that the amine of the serine headgroup coordinates with La^{3+} at pH 7.4. The excitation spectrum of 2 mM DMPS and 0.13 mM Tb^{3+} in 0.1 M NaCl was collected at several pH values between 3 and 11.5 at 50°C. Three distinct changes occur in the Tb^{3+}-DMPS excitation spectrum over this pH range (Fig. 4): (*a*) pH 3.1–5.5: a 234-nm band grows at the expense of a band at 245 nm; (*b*) pH 6.1–9.0: a band grows in at ~238 nm which as an amplitude an order of magnitude greater than the dominant bands at lower pH; (*c*) pH 9.0–11.5: the 238-nm band gradually shifts to 245 nm and decreases in amplitude while a very broad band grows in at ~308 nm.

There are more subtle changes occurring in the many lower amplitude bands which will not be discussed here. The 234, 238, and 245-nm bands arise from 4f to 5d transitions while the 308 nm may be either a 4f to 5d or a charge transfer transition. Below pH 7, aquo-Tb^{3+} has two 4f to 5d excitation bands in the region studies, one at 263 nm and another at 217 nm(Carnall et al., 1968; Halladay and Petersheim, 1988). The latter actually appears at 225 nm with this instrument because the lamp intensity drops off rapidly below 230 nm. Because neither aquo-Tb^{3+} band is observed in the DMPS solutions, all of the Tb^{3+} is bound to lipid. Above pH 7, the aquo species forms complexes with hydroxide ions, which results in the original two 4f-to-5d bands being replaced by a single dominant band at 238 nm. The spectrum of Tb^{3+}-DMPS at pH 9 (Fig. 4) has a band in this position but the emission intensity is two orders of magnitude greater than that from the aquo-hydroxide complex, indicating that even if a hydroxide complex is formed, it is also bound to DMPS.

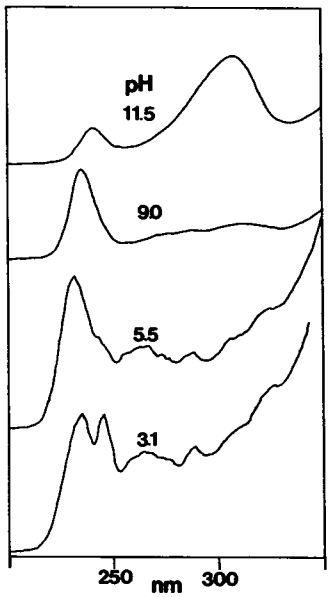

FIGURE 4 pH dependence of the Tb^{3+}-DMPS excitation spectrum. All spectra are for the same sample of 2 mM DMPS and 0.125 mM Tb^{3+} in 0.1 M NaCl with the pH adjusted with either HCl or NaOH. Emission was monitored at 545 nm and the spectra have not been corrected for lamp and monochromator profiles. The detector gain for the pH 3.1 and 5.5 spectra is 10 times that of the other two and the sharply sloping baseline at higher wavelengths is due to light scattering by the lipid vesicles. Spectra were also collected at pH 3.7, 4.2, 4.8, 6.1, 7.4, 8.4, 10.3, and 11.0. The midpoints for the spectral changes are at approximately pH 4.0, 7.4, and 11.

In 0.1 M NaCl, the apparent pK_a for the phosphatidylserine carboxylate is 5.5 (Cevc and Marsh, 1987), thus the loss of the 245-nm band between pH 3.1 and 5.5 reflects deprontonation of the DMPS carboxylic acid group and coordination of Tb^{3+} by the carboxylate anion. The 234-nm band at pH 5.5, then, is indicative of a phosphate-carboxylate chelate of Tb^{3+}. The 245-nm band undoubtedly reflects coordination by the phosphate but it is not clear whether the protonated carboxylic acid is also involved in coordination. Between pH 7.4 and 9.0 the growth of the 238-nm band is consistent with formation of $Tb(OH)^{2+}$ which complexes with the phosphate and carboxylate of DMPS. Finally, the growth of the 308-nm band coincides with deprotonation of the ammonium group (apparent pKa = 11.5; Cevc and Marsh, 1987), and coordination by the primary amine. It is possible that this dominant low energy band results from charge transfer from the amine nitrogen to Tb^{3+}. Coordination by the amine is apparently not significant until about pH 10, where there is first evidence of a strong 308-nm band. The phosphate group is probably involved in coordination at all times.

These results and their interpretation are consistent with all of the relevant observations made by Bentz et al. (1988). That is, their observed drop in pH when La^{3+} is added to a phosphatidylserine suspension at pH 7.4 and the change in liposome zeta potential at high pH could be due to $La(OH)^{2+}$ forming and binding to the lipid rather than deprotonation of the ammonium group. The pH drop observed for La^{3+} binding at pH 4.5 is probably due to the La^{3+} forcing deprotonation of the serine carboxylate.

CONCLUSION

The observations presented here indicate that La^{3+} is coordinated by the carboxylate and phosphate moieties of phosphatidylserine above pH 4, and that the serine amine is probably not involved in the complex below pH 10. The bound cation causes a conformational change in the phosphodiester which is very likely the same as that observed for Ca^{2+} binding to phosphatidylserine (Casa et al., 1987). This conformational change is not a direct consequence of forming the cation-phosphatidylserine complex, but appears to be a more delocalized effect of the cation on headgroup packing, i.e., electrostatic repulsion of the serine ammonium group. This statement is supported by the observation that the phosphate resonance representing the outer surface of the DMPS vesicles requires a relatively high level of bound La^{3+} (1:4 La^{3+}:DMPS) before a measurable shift occurs. The more facile response of the inner surface headgroups to La^{3+} binding is an indication that the conformational change alleviates some of the headgroup crowding on the inside.

Given these observations and the effect of cation binding on phosphatidylcholine membranes (McIntosh, 1980; Seelig et al., 1987), it seems likely that below pH 10 the binding of Ca^{2+} and La^{3+} forces the serine ammonium group away from the membrane surface by attenuating the negative surface charge or even generating a net positive charge. If there are tight ionic bridges formed in the fusion intermediate for phosphatidylserine vesicles, as suggested by Bentz et al. (1988), the serine ammonium group may be playing a direct role with the cation-induced conformational change making it more available for bridging with an adjacent membrane. This interaction does not preclude direct involvement of the bound cations in other ionic bridges. Both Ca^{2+} and La^{3+} can have coordination numbers greater than 6. Therefore, half of the ligand sites remain available even with coordination by both the phosphate and carboxylate of one phosphatidylserine. The fact that both cations bind to the phosphodiester group simply requires a very tight association of the two adjacent membranes before they can be involved in bridging. Optical studies of Tb^{3+}-induced fusion will provide more direct evidence for the chemical nature of these fusion intermediates.

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