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^2H and ^{31}P NMR study of pentalysine interaction with headgroup deuterated phosphatidylcholine and phosphatidylserine

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Abstract. The interaction of cationic pentalysine with phospholipid membranes was studied by using phosphorus and deuterium Nuclear Magnetic Resonance (NMR) of headgroup deuterated dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylserine (DMPS). In the absence of pentalysine, some of the deuterium and phosphorus spectra of DMPC/DMPS 5:1 (m:m) membranes gave lineshapes similar to those of partially-oriented bilayers with the planes of the bilayers being parallel to the magnetic field. The deuterium NMR data show that the quadrupolar splittings of the deuterated methylenes of the DMPC headgroup are not affected by adsorption of pentalysine on the PC/PS membranes. By contrast, the pentalysine produces significant changes in the quadrupolar splittings of the negatively charged DMPS headgroup. The results are discussed in relation to previous ^2H NMR investigations of phospholipid headgroup perturbations arising from bilayer interaction with cationic molecules.

Key words: Phosphatidylserine, polylysine, lipid-peptide interaction, deuterium NMR, phosphorus NMR

Introduction

A comprehensive approach to membrane/protein interactions requires detailed studies at the molecular level of the lipid and protein components. In this respect, broad-line deuterium (^2H) NMR[1] is already established as a valuable tool, since it is well suited to provide fundamental structural and thermodynamic information on anisotropic membrane structures. An attractive feature of ^2H NMR, is that the selective deuteration of phospholipid molecules allows the study of a particular class of lipid (PC, PE, PS, PG ...) at specific sites, located either in the polar region of the bilayer or in the hydrophobic core.

Most of the previous ^2H NMR studies of protein/lipid interactions have been concerned with zwitterionic phospholipids (PC, PE) mainly deuterated on the fatty acyl chains (for a review see Seelig and Seelig 1980; Devaux 1983; Davis 1983; Bloom and Smith 1985), and more rarely the polar moiety (Tamm and Seelig 1983; Dempsey et al. 1986; Ryba et al. 1986). The general feature of these experiments, is that the incorporation of large integral membrane proteins such as cytochrome oxidase (Tamm and Seelig 1983), rhodopsin (Bienvenue et al. 1982) or Ca^{2+} ATPase (Seelig et al. 1981) does not produce significant variations of the deuteron order parameter, although a consequent broadening of the resonance line could be detected. Recently several laboratories have undertaken the investigation of the interaction between proteins and anionic lipids such as PG and PS. These studies have indicated that the polar moiety of negatively charged lipid is quite sensitive to the membrane interaction with amphiphilic peptides (Sixl and Watts 1985) and peripheral proteins (Sixl et al. 1984). Devaux et al. (1986) have reported ^2H NMR results concerning the interaction of PS with cytochrome c, leading to the conclusion that the fatty acyl chain order of DMPS was not significantly perturbed, but that one of the quadrupolar splittings of the PS headgroup deuterons was affected to a measurable degree. Most ^2H NMR studies of protein/lipid interactions have involved reconstituted systems with large natural membrane proteins, and there is a lack of available data obtained from simple well-defined model systems. Polylysines have been used as models of extrinsic protein (De

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Abbreviations: NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; POPC, 1-palmitoyl, 2-oleyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl, 2-oleyl-*sn*-glycero-3-phosphoglycerol; PC, phosphatidylcholine; PS, phosphatidyl serine; PG, phosphatidylglycerol; HEPES, *N*-(2-hydroxy-ethyl)piperazine-*N*'-2-ethanesulfonic acid; TRIS, *tris*-(hydroxymethyl)aminoethane; EDTA, ethylenediamine-tetra-acetic acid.

Kruijff and Cullis 1980; Hartmann and Galla 1978). The present paper describes experiments performed with pentalysine. In the experiments reported here, DMPS was diluted in DMPC bilayers, in order to increase the peptide/PS ratio while maintaining a reasonable total peptide to lipid ratio. Such a procedure, leading to a decrease of free (non-interacting) PS, should i) optimize the detection of a PS/peptide complex, if it exists ii) facilitate the separation of specific lipid/peptide interactions from bulk lipid phase perturbations by "control" experiments with headgroup deuterated DMPC.

Materials and methods

Lipids and peptides

DMPC was purchased from SIGMA and pentalysine (acetate form) from BACHEM. Headgroup deuterated DMPC and DMPS were, prepared according to the procedures described by Roux et al. (1983) and Roux and Neumann (1986), respectively. In the following, these compounds will be denoted as:

$O(O)P(O)O-CD_2-CH_2-N(CH_3)_3$	DMPC$\alpha-CD_2$
$O(O)P(O)O-CH_2-CD_2-N(CH_3)_3$	DMPC$\beta-CD_2$
$O(O)P(O)O-CD_2-CH-(NH_2)COOH$	DMPS$\alpha-CD_2$
$O(O)P(O)O-CH_2-CD-(NH_2)COOH$	DMPS$\beta-CD$

Binding experiments

The sample preparation was the same as those used for the NMR experiments, except that the dried lipid mixtures (6.25 μmol) were dispersed in *Tris* buffer (50 m*M*, pH 7.5, 40 m*M* NaCl, 1 m*M* EDTA) containing appropriate amounts of pentalysine. The final volume of added buffer was 200 μl, leading to a lipid concentration of 37.5 m*M*. After the centrifugation step, the amount of bound pentalysine was determined from the amount of free peptide remaining in the supernatant as determined by the Lowry assay (optical absorption at 750 nm) (Lowry et al. 1951). *Tris* buffer was used in order to avoid the side reaction of the Hepes buffer occurring in the Lowry assay.

Sample for NMR experiments

Liposomes were prepared by mixing appropriate amounts of DMPC (93 mg in chloroform) and DMPS (20 mg in chloroform/methanol 9:1). Solvents were removed by evaporation under reduced pressure and the solid residue dried under high-vaccum for 15 min. The residue was then dissolved in pure chloroform, the solvent evaporated, and the resulting lipids dried under high vacuum (10^{-2} mm Hg) for 12 h. The lipids were then dispersed in 200 μl Hepes buffer (50 m*M* in deuterium depleted water, pH 7.5, 40 m*M* NaCl, 1 m*M* EDTA) at 50 °C, with continuous vortexing. After stepwise addition of 3.8 ml of excess buffer (total volume 4 ml), the resulting samples were submitted to five freezing (liquid nitrogen) and thawing (50 °C) cycles, and centrifuged at 30 °C (45,000 rpm, 2 h), NMR experiments were then carried out on the pellet. The concentration of total lipid in the pellet was about 150 m*M*. The pentalysine-containing samples were prepared by lyophilization of the pellet, followed by rehydration of the dry lipid powder with the same volume of pure deuterium-depleted water, containing appropriate amounts of peptide. The resulting suspensions were then submitted to extensive freezing-and-thawing, and transferred into the NMR tube (Method 1). Some samples were also prepared without lyophilization, by simply adding an appropriate amount of a buffer solution of the peptide to the pellet, followed by extensive freezing-and-thawing (Method 2). All the peptide-containing samples were prepared by method 1 unless otherwise stated in the text.

NMR experiments

^{2}H NMR experiments were done at 46 MHz on a Bruker MSL-300 spectrometer with a spectral width of 83.3 kHz. A quadrupolar echo pulse sequence (Davis et al. 1976) was employed with a repetition time of 100 ms. The pulse length and the pulse separation were respectively 5.5 μs and 120 μs. A home-built ^{2}H NMR spectrometer described elsewhere (Davis 1979) was also used, with a spectral width of 250 kHz and a pulse separation and recycling delay of respectively 3 μs, 50 μs and 125 ms. Deuterium spectra were recorded with the Bruker MSL-300 unless otherwise stated in the text.

The ^{2}H NMR signals were shifted by some fraction of the dwell time to ensure that the Fourier Transform started precisely on top of the quadrupolar echo (Davis et al. 1976). ^{31}P NMR experiments were run at 81 MHz (Bruker WP-200) in 15 mm diameter tubes with proton broadband decoupling of 2 W raised to 8 W during the acquisition. We used a spectral width of 31.2 kHz, with a single 90° pulse of 15 μs and a recycling time of 1 s.

Results

Binding studies

The binding data of pentalysine obtained with DMPC and DMPC:DMPS 5:1 liposomes, are displayed in

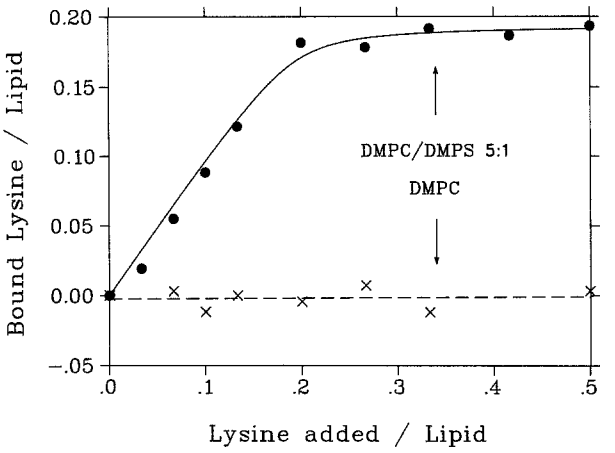

Fig. 1. Binding of pentalysine to DMPC (*cross*) and DMPC/DMPS 5:1 (m:m) (*filled circle*) as a function of the amount of peptide present in the lipid dispersion. The *solid curve* is the result of a fit (see discussion) done with the data depicted by the *filled circle*. The units are expressed in lysine monomer per lipid molecule. The total lipid concentration was 37.5 m*M*

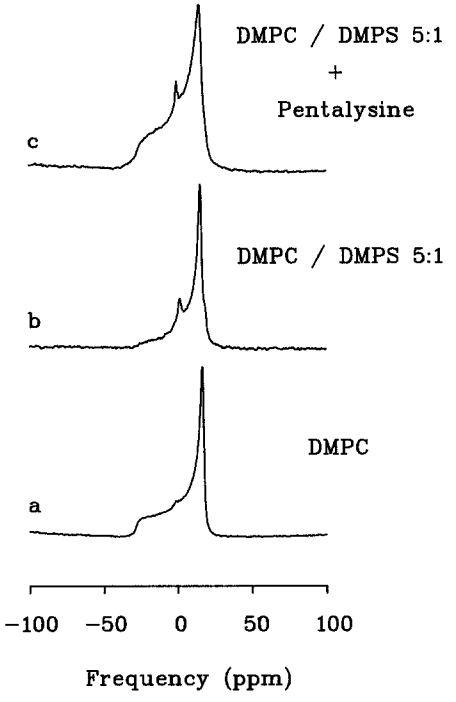

Fig. 2 a–c. ^{31}P NMR spectra of pure DMPC bilayers (**a**), and DMPC DMPS/5:1 (m:m) bilayers recorded either in the absence (**b**) or in the presence (**c**) of pentalysine with a lipid to peptide molar ratio of 6:1.5. Measuring temperature was 34 °C

Fig. 1 with a plot of the amount of membrane-bound pentalysine versus the total amount of peptide present in the membrane suspension. The units were obtained by calculating the amounts of bound and total lysine monomer per lipid molecule. A significative binding of pentalysine is observed only with DMPS-containing

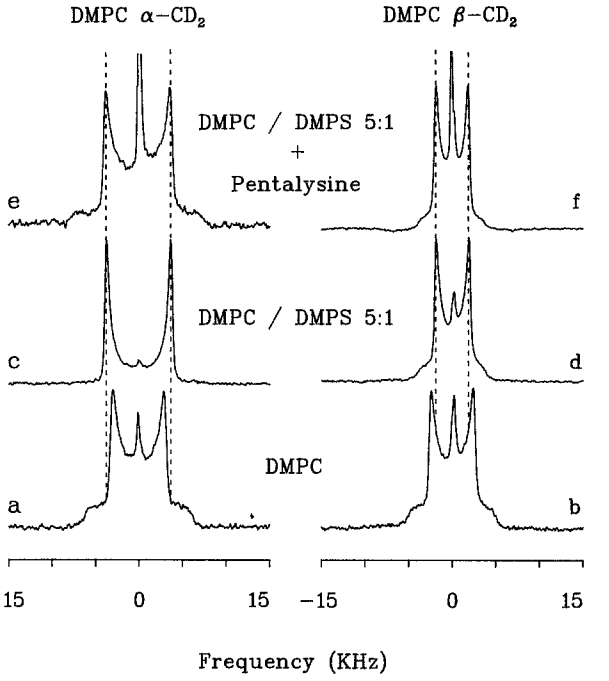

Fig. 3 a–f. ^{2}H NMR spectra at 46 MHz of DMPC α-CD_2 (**a, c, e**) and β-CD_2 (**b, d, f**) recorded from DMPC (**a, b**) or DMPC/DMPS 5:1 (m:m) bilayers either in the absence (**c, d**) or the presence (**e, f**) of pentalysine with a lipid to peptide molar ratio of 6:1.5. Measuring temperature was 34 °C

liposome. The binding curve displayed in Fig. 1 for the DMPC/DMPS 5:1 membranes show, that within the experimental error, a plateau is observed for a lysine to lipid ratio close to 0.2, corresponding to a lysine monomer to PS ratio of 1.2. Figure 1 also shows that for a total lysine to lipid ratio of 0.5 (0.6 pentalysine per DMPS), the saturation conditions are almost achieved.

^{31}P *NMR experiments*

Pure DMPC membranes exhibit a typical axially symmetric powder spectrum (Fig. 2a). The spectral lineshape (Fig. 2b) of the negatively charged membranes of DMPC/DMPS 5:1 (m:m) is different insofar as the resonance intensities of the low-field shoulder are decreased. The incorporation of pentalysine (Fig. 2c) results in a more classical bilayer pattern. Within the limits of the experimental resolution, the chemical shift anisotropies are not changed by the incorporation of DMPS either in absence or presence of pentalysine.

Headgroup deuterated DMPC

Figure 3 displays ^{2}H NMR spectra (46 MHz) of headgroup deuterated DMPC either pure or mixed with

DMPS and pentalysine. The spectra of Fig. 3 a and b recorded from pure DMPC membranes exhibit the standard feature of Pake doublet powder spectra arising from a superposition of randomly oriented bilayers. The two sharp features, often called the "edges" of the spectrum arise from bilayers whose normals are perpendicular to the external magnetic field, i.e. those for which the magnetic field is parallel to the bilayer surface. Their separation defines the "quadrupolar splitting" of the spectrum. The weaker rounded features which are separated by twice the splitting are called the "shoulders" of the spectrum. The deuterium spectrum of the α-CD_2 methylene group, recorded with the DMPC/DMPS 5:1 membranes in the absence of pentalysine (Spectrum c), is appreciably different from a typical powder lineshape. The intensities of the shoulders are considerably lower than those of a classical powder type spectrum. This effect is less pronounced for the DMPC β-CD_2 (Spectrum d). This particular result may be due to a lack of reproducibility in the sample preparation. Other experiments performed with acyl chains perdeuterated DMPC/DMPS membranes (M. Roux, unpublished results) show that classical powder pattern and lineshape with reduced shoulders may both be obtained, depending on the preparation procedure. Classical powder lineshapes are clearly observed for both methylene groups with pentalysine containing samples (Spectra e, f). The large isotropic peaks occurring for these samples, which are less important in the corresponding phosphorus spectra, are mainly due to the natural abundance of deuterium in water. As shown in Table 1, the incorporation in DMPC bilayer of 0.2 mol DMPS induce an increase (24%) and a decrease (21%) of the α-CD_2 and β-CD_2 quadrupolar splittings, respectively. The main result is that no variations of either the DMPC α and β quadrupolar splittings are detected after addition of pentalysine to the mixed DMPC/DMPS bilayers.

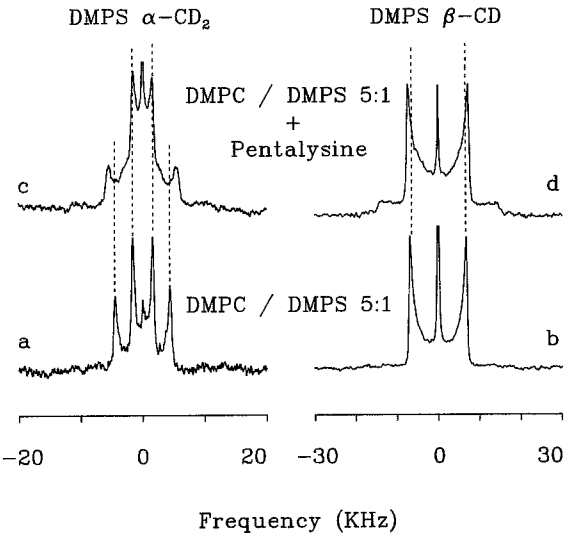

Fig. 4 a–d. ^2H NMR spectra at 46 MHz of DMPS α-CD_2 (**a, c**) and β-CD (**b, d**) in bilayers of DMPC/DMPS 5:1 (m:m), recorded either in the absence (**a, b**) or the presence (**c, d**) of pentalysine with a lipid to peptide molar ratio of 6:1.5. Measuring temperature was 34 °C. NMR spectra of DMPS β-CD were obtained with the home-built spectrometer (see Materials and methods)

Table 1. Deuterium quadrupolar splittings (KHz) of headgroup deuterated DMPC and DMPS obtained by peak-to-peak measurements from the ^2H NMR spectra

	DMPC:DMPS:Pentalysine mole ratio			
	5:0:0	5:1:0	5:1:0.15	5:1:1.5
DMPC				
α-CD_2	5.8	7.3	–	7.3
β-CD_2	4.8	3.8	–	3.6
DMPS				
α-CD_2 external		9.0	10.5	11.0
α-CD_2 internal		3.1	3.0	3.0
β-CD		13.6	–	14.6

Headgroup deuterated DMPS

Deuterium NMR spectra of headgroup deuterated Phosphatidylserine in the bilayer state, show two distinct quadrupolar splittings for the α-CD_2 methylene (9 and 3.1 kHz) (Fig. 4 a) and one splitting for the β group (13.6 kHz) (Fig. 4 b). The occurrence of two Pake doublets for the α methylene is due to the inequivalence of the two deuterons of the CD_2 group (Browning and Seelig 1980). In agreement with previous observations (Browning and Seelig 1980; Roux and Neumann 1986), the deuterons of both the α and β group of the serine moiety exhibit smaller quadrupolar splittings in bilayers containing phosphatidylcholine than in pure phosphatidylserine membranes. The shape of NMR spectra of the DMPS headgroup in mixed DMPC/DMPS bilayers (Fig. 4 a and b) are similar to those of DMPC headgroup presented above (Fig. 3 c) i.e. the external shoulders are not clearly detected. The incorporation of pentalysine in the DMPC/DMPS 5:1 (m:m) affects both the size of the quadrupolar splittings and the lineshape of the NMR spectra (Fig. 4 c and d, and Table 1). The value of the β-CD and external α-CD_2 quadrupolar splittings measured in the presence of an excess of pentalysine (1.5 mol for 1 mol PS), are respectively 14.6 and 11 kHz and are higher than those observed in the absence of the peptide. The internal quadrupolar splitting is not affected by the addition of pentalysine. Intermediate concentrations of pentalysine give α-CD_2 spectra similar to those displayed in Fig. 4 c, although

the external quadrupolar splitting is smaller (10.5 kHz for a peptide/PS ratio of 0.15, data not shown). The NMR spectra of DMPS head-group deuterons recorded after addition of pentalysine gave more classical Pake doublet powder lineshapes, in particular the shoulders are clearly detected, for both lyophilized (Method 1) (Spectra c, d) and unlyophilized samples (Method 2) (not shown).

Discussion

Polylysines do not bind to zwitterionic membranes such as pure phosphatidylcholine bilayers. De Kruijff et al. (1985) showed that polylysines were able to bind to negative cardiolipin-containing membranes only if the peptide molecules consist of at least three monomers ($n=3$). These authors showed that the dissociation constant of pentalysine ($n=5$) for cardiolipin was 0.18 mM, and observed a plateau for about two lysine residues per cardiolipin molecule, corresponding to an equimolar amount of positive and negative charges. A quantitative analysis of our PC/PS binding data[1] leads to a dissociation constant K_d^{app} of 28 (± 18) μM, and to a value of 26 (± 1) lipids by peptide in the saturation limit, i.e. one pentalysine binding site should contains 21.7 DMPC and 4.3 DMPS. Such a picture of the pentalysine binding site seems consistent if we consider that one pentalysine molecule contains five net positive charges and should therefore be able to bind five molecules of DMPS. Our binding studies show also that saturation is almost obtained for a pentalysine to PS ratio of about 0.5 indicating that, in these conditions, most of the PS binding sites are occupied. It is thus very likely that the NMR data presented here were recorded under saturation conditions since the DMPC/DMPS 5:1 samples used for the experiments contained 1.5 pentalysine per DMPS molecule.

The addition of pentalysine to DMPC/DMPS 5:1 (m:m) membranes affects lineshapes of both phosphorus and deuterium spectra of either PC or PS deuterated headgroup, by cancelling the 90° edge enhancement observed with such membranes in the absence of peptide. ^{31}P and ^{2}H NMR lineshapes similar to those obtained with the DMPC/DMPS 5:1 (m:m) dispersions in the absence of pentalysine, have already been reported for POPE/POPG (Seelig et al. 1985) and sphingomyelin (Speyer et al. 1987) membranes. In both cases, the authors claimed that these particular lineshapes were due to a magnetic orientation of the phospholipid bilayers. A preferred bilayer alignment with the membrane surface parallel to the magnetic field would induce a simultaneous increase of the 90° peak and decrease of the 0° shoulder of the bilayer pattern, and therefore lead to NMR spectra similar to those obtained for the DMPC/DMPS 5:1 (m:m) lipid mixture. Indeed, the fact that these non-bilayer lineshape are observed at the same time for the ^{31}P NMR spectrum and almost all ^{2}H NMR spectra of deuterated headgroups of DMPC/DMPS 5:1 membranes, suggests that these effects are related to a macroscopic perturbation of the bilayers. Magnetic field-induced orientation of phospholipid membranes has been clearly demonstrated by microscopy experiments (Boroske and Helfrich 1978). The observations were consistent with the assumption that magnetic orientation arose from the negative anisotropic susceptibility of the lipid molecules, resulting in a preferred orientation of the hydrophobic chains perpendicular to the direction of the magnetic field. Since spherical vesicles cannot give any preferred orientation, membrane alignment is related to the bilayer ability to adopt a non-spherical shape (Speyer et al. 1987). Atkinson et al. (1974) have shown that due to repulsive interactions at low ionic strength, negative phosphatidylserine bilayers exhibit a pronounced swelling (i.e. continuous hydration), leading to large quasi-unilamellar vesicles. Thus, the incorporation of negatively charged DMPS in DMPC bilayers may likewise induce the formation of such vesicles, which, because of their "flexible" structure, could more readily be oriented in the magnetic field than "stacked" neutral liposomes of pure DMPC. In this context, the pentalysine-induced "non-oriented" powder lineshape may be due to a screening effect of this peptide upon the negatively charged bilayer, leading to a reduction of the water layer and a formation of multilamellar liposomes. Cation-induced screening effects of negatively charged PS bilayers have been observed previously and discussed in X-ray (Hauser and Shipley 1983) and NMR studies (Roux and Neumann 1986). At this point, we wish to underline that an orientation dependence of T_2 could also lead to an increase of the edge intensity relative to the shoulders. Moreover, Bloom and Sternin (1987) have shown that in DPPC bilayers, the deuterium T_2 measured on the acyl chains were affected by slow motions probably related to the lateral diffusion of the lipid molecules around the membrane surface. Thus, one could also expect that the macroscopic changes such as those observed with charged bilayers

[1] Such an analysis was achieved by a Gauss-Newton fit (Daniel and Wood 1971) of the data, by the expression:

$$K_d^{app} = \frac{[M][P]}{[MP]},$$

where [P] and [MP] are respectively the experimental concentrations of free and bound peptides. The concentration of free "membrane sites" [M] is defined by [M]=[L]/n, where [L] is the lipid concentration and the n the number of lipids per site (Devaux and Seigneuret 1985). The fitted parameters are K_d^{app} and n.

may influence the T_2 of the lipid deuterons. At the present time, the influence of an orientation dependence of T_2 in the perturbations of both ^{31}P and ^{2}H NMR spectral lineshapes cannot be ruled out.

In any case, the lineshape changes described above have a negligible influence on the determination of the deuterium quadrupolar splittings of the DMPC and DMPS headgroups. It is remarkable that the choline headgroup splittings remain unchanged upon binding of pentalysine to phosphatidylcholine-containing membranes. These results are surprising since previous investigations have shown that the quadrupolar splittings of the choline headgroup are very sensitive to the presence of charged species such as ions (Akutsu and Seelig 1981; Altenbach and Seelig 1985), tetracaine (Boulanger et al. 1981) or acidic phospholipids (Sixl and Watts 1983). Seelig et al. 1987 have discussed the sensitivity of the PC headgroup to these various charged molecules, in terms of electrostatic effects of their net charges, on the choline dipole. In this context, the headgroup quadrupolar splittings of DMPC should have been also affected by the cationic charges of membrane-bound pentalysine. Pentalysine interacts externally with the membrane surface, and the lack of variation of the PC splittings observed in the presence of this peptide may be due to a weaker bilayer penetration of the lysine cationic groups inside the bilayer. Indeed, the hydrophobic core of tetracaine and anionic lipids lead them to partition into zwitterionic PC bilayers, and to interact closely, *from the inside of the bilayer*, with the choline headgroup. Likewise, small inorganic cations, which are already able to bind to pure PC membranes (Cunningham et al. 1986 and references therein), may penetrate more deeply into the bilayer than bulky peptide molecules.

The PS headgroup is expected to be more sensitive to the binding of pentalysine than the choline moiety, since the binding of this peptide requires the presence of negatively charged lipid such as the PS molecule. As shown by the binding studies, each DMPS molecule should interact with almost one pentalysine NH_3^+ group under the conditions used during the NMR experiments. Yet, the deuterium NMR spectra recorded from headgroup deuterated DMPS do not indicate dramatic changes of the quadrupolar splittings of the serine headgroup. The spectra displayed in Fig. 4 do not reveal the presence of two components in slow exchange, so that any hypothetical PS/pentalysine complexes are short lived on the NMR time-scale. As in the study of Devaux et al. (1986) with cytochrome c, only one of the α-CD_2 quadrupolar splittings is modified by the addition of peptide to DMPC/DMPS 5:1 (m:m). It is interesting to note that the results obtained with cationic pentalysine are quite different from those observed after adsorption of lithium ions at the surface of PC/PS membranes, for which a large reduction (40%) of the internal α splitting was obtained (Roux and Neumann 1986). Thus, the interaction of the PS head-group with the small cation Li^+, induces larger splitting perturbations than those detected in the presence of a large peptidic molecule. More experimental studies are required on PS headgroup, in order that the results obtained with pentalysine or other charged peptides may be properly interpreted in terms of molecular reorganization of the phospholipid headgroups.

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