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A NMR investigation on the interactions of the α-oligomeric form of the M13 coat protein with lipids, which mimic the *Escherichia coli* inner membrane

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The interaction of the M13 bacteriophage major coat protein in the α-oligomeric form with specifically deuterated phospholipid headgroups which mimic the *Escherichia coli* inner membrane, has been studied using NMR methods. As can be seen from the deuterium NMR spectra obtained with headgroup trimethyl deuterated DOPC, the coat protein in the α-oligomeric form does not give rise to trapped lipids as observed with M13 coat protein in the β-polymeric form (Van Gorkom et al. (1990) Biochemistry 29, 3828–3834). The quadrupolar splittings of the α headgroup methylene deuterons of deuterated phosphatidylcholine and phosphatidylethanolamine decrease, whereas the quadrupolar splittings of the β headgroup methylene deuterons of the two lipids increase with increasing protein content. All deuterated segments in the phosphatidylglycerol headgroup show the same relative decrease of the NMR quadrupolar splittings. These results are interpreted in terms of a change in torsion angles of the methylene groups, induced by positive charges, probably lysine residues of the protein at the membrane surface. For all lipid bilayer compositions studied the head-group perturbations are similar. It is concluded that there is no strong specific interaction between one of the lipid types examined and the M13 coat protein. From the spin-spin (T_{2e}) relaxation time and spin-lattice (T_{1z}) relaxation time of all deuterated lipids it is concluded that at the bilayer surface only slow motions are affected by the M13 coat protein.

Introduction

It has been shown that in model membranes M13 coat protein (mol. wt. 5240; for reviews, see Refs. 2 and 3) can adopt two different conformations, which depend critically on the headgroup type and the degree of unsaturation of the acyl chains [4,5,6]. The two forms of the M13 coat protein differ in their α-helix content and also show differences in the aggregation state of the protein. The protein in the α-oligomeric form has been shown to be in a less aggregated state than the protein in the β-polymeric form [6]. The protein in the α-oligomeric form has oligomers of less than 25 subunits [6] and can be obtained directly from the phage particle or when folded from a random coil conformation [4,7].

In this investigation we have studied M13 coat protein in the α-oligomeric form and its interaction with lipid bilayers which mimic the *E. coli* inner membrane. The composition of the inner membrane of *E. coli*, which is normally 74% PE, 19% PG and 4% CL, is changed during infection when the relative level of PE decreases in preference to an increase in the relative concentration of the lipids PG and CL [8]. This NMR

Abbreviations: CD, circular dichroism; CL, cardiolipin; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPG, dioleoylphosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; L/P, lipid to protein molar ratio; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; T_{1z}, spin lattice relaxation time; T_{2e}, deuterium quadrupolar echo spin-spin relaxation time; T_2, spin-spin relaxation time; Tris, tris(hydroxymethyl)aminomethane hydrochloride.

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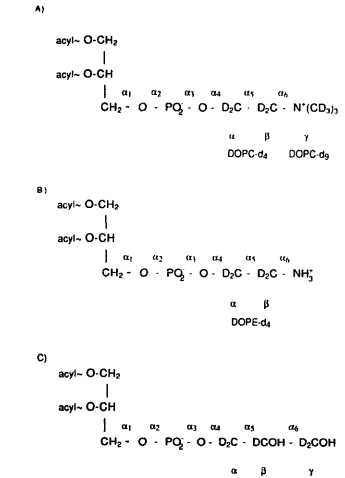

Fig. 1. Chemical formula of (A) DOPC labelled at various positions in the headgroup region (DOPC-d_4 and DOPC-d_9); (B) DOPE labelled in the headgroup (DOPE-d_4); (C) DOPG labelled in the headgroup (DOPG-d_5).

investigation was performed to investigate how M13 coat protein interacts with one of the lipid bilayer components of the *E. coli* inner membrane. To mimic the *E. coli* inner membrane mixtures of the phospholipids DOPC, DOPE, DOPG and CL were used. DOPC was specifically deuterated either in the headgroup at both the α and β-methylene segments (DOPC-d_4) or at the trimethyl moiety (DOPC-d_9, Fig. 1A). DOPE was deuterated at both the α- and β-methylene segments of the choline headgroup (Fig. 1B) and DOPG-d_5 was labelled at the α, β and γ segments (Fig. 1C). In addition a comparison will be made with the results previously obtained with the protein in the β-polymeric form [1,9,10–15].

Materials and Methods

Lipid synthesis. DOPC-d_9 deuterated in the trimethyl groups of the choline moiety, was synthesized from DOPE as described by Eibl [16]. The synthesis of 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-glycerol, perdeuterated in the glycerol headgroup, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, both deuterated in the α and β-methelene segments has been described before [17]. After purification, the lipids appeared as one spot on high performance silica TLC (solvent $CHCl_3$, MeOH, NH_4OH, 55:30:3, v/v/v) and were stored at −20 °C. DOPE, DOPC, DOPG and CL were obtained from Sigma (St. Louis, U.S.A.) and used without any further purification.

Protein purification and reconstitution. Bacteriophage M13 was grown and purified as described previously [6]. The desired amounts of lipids (40 mg for DOPC-d_9, 75 mg for DOPC-d_4, 75 mg for DOPE-d_4 and 75 mg for DOPG-d_5) were mixed with the desired amount of unlabelled lipids in chloroform. After removing the chloroform with nitrogen gas, samples were lyophilized for at least 12 hours and solubulized in buffer (50 mM cholate (Sigma), 10 mM Tris (Sigma), 0.2 mM EDTA (Sigma), 140 mM NaCl, pH 8.0) by sonication (Branson B15, duty cycle 50%, 40 W). To this solution the desired amount of protein in the same buffer was added followed by dialysis at room temperature against 100-fold excess buffer (10 mM Tris, 0.2 mM EDTA, 140 mM NaCl, pH 8.0) for a total of 48 h changing the buffer every 12 h. The reconstituted lipid-protein complex was concentrated using an Amicon stirring cell to 500 μl and lyophilized for at least 12 h and resuspended in 500 μl of deuterium depleted water (Sigma). The L/P ratio of each sample was determined by a phosphate assay with AMP as a standard [18] and a modified Lowry procedure to determine the protein content, using BSA as a standard [19]. To check sample homogeneity, aliquots were layered on a linear 0–40% w/w sucrose gradient and centrifuged at 100 000 × g for 16 h at 5°C. The homogeneity was checked visually. For CD measurements, samples were diluted to a protein concentration of 0.1 mg/ml and an average of at least five scans were recorded at room temperature on a Jovin-Ivon Dichograph Mark in in the wavelength range 200–250 nm, using a 0.1 cm path length. The spectra were analyzed using reference spectra of Greenfeld and Fasman (1969). The aggregation state of M13 coat protein was checked by HPLC as described previously [6].

NMR measurements. All NMR spectra were recorded on a Bruker CXP 300 spectrometer. The 46.1 MHz ^{2}H-NMR spectra and T_{2e} relaxation times were recorded using a quadrupolar echo pulse sequence, using quadrature detection (90°_x-τ-90°_y-τ-acq.) with full phase cycling [21]. The T_{2e} relaxation times were determined by plotting the intensity of the echo peak as a function of 2τ, where τ is the separation between the two pulses. The T_{1z} relaxation times were recorded using an inversion recovery method in combination with the quadrupolar echo sequence (180°_x-τ_2-90°_x-τ_1-90°_y-τ_1-acq.) and the relaxation times were determined as a function of the echo intensity versus τ_2. Typical

instrumental parameters were: spectral width of 100 kHz, 90° pulse width of 5 μs and relaxation delay of 250 ms. 121.4 MHz ^{31}P-NMR spectra and T_2 relaxation times were recorded with broadband decoupling and a Hahn-echo sequence (90°_x-τ-180°_y-τ-acq.) using full phase cycling [22] Typical instrumental parameters for ^{31}P-NMR were spectral widtli 100 kHz, 90° pulse width of 5 μs and relaxation delay of 2.5 s. The temperature was controlled with a nitrogen gas flow unit and measured with an accuracy of ±1 C°. Oriented spectra were obtained numerically from the experimental spectra by using an iterative depaking program [23]. The quadrupolar splittings given in the tables are all obtained from these oriented spectra.

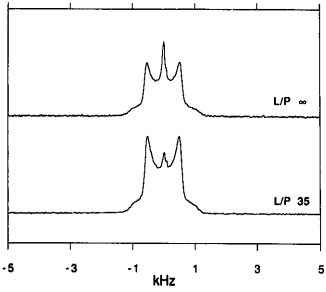

Fig. 2. 46 MHz ^{2}H-NMR spectra of DOPC-d_9 at 25°C (A) L/P ∞ and (B) L/P 35. Experimental parameters as described in the text.

Results

All the samples were checked for their homogeneity and for the conformation and aggregation state of the protein. Sucrose gradients of all samples showed only one band indicating that the samples were homogeneous. CD spectroscopy and HPLC elution profiles indicated that the protein was in the α-oligomeric form, with a predominant α-helix conformation and no indication of strong protein aggregation [6]. All the spectra containing DOPE were recorded at 5°C. No indication was found for the presence of phospholipids in a hexagonal phase by ^{31}P-NMR spectra.

The quadrupolar splitting of the γ headgroup segment of DOPC-d_9 is independent of the L/P ratio. A typical example of the spectra obtained from DOPC-d_9 is shown in Fig. 2. The T_{2e} relaxation time of DOPC-d_9 decreases with increasing protein concentration whereas the T_{1z} relaxation time is independent of the L/P ratio (Table I).

The ^{2}H-NMR spectra of DOPC-d_4 bilayers with L/P ratios of ∞ and 35 are shown in Fig. 3A. The spectra are a superposition of two powder patterns from the α headgroup and β headgroup deuterons. The outer splitting is attributed to the α headgroup deuterons and the inner component is attributed to the β headgroup deuterons [24]. The equivalent oriented spectra obtained by depaking clearly show changes in α and β segment splittings in the presence of protein. The splitting of the β segment increases with decreasing L/P ratio, in addition a corresponding decrease of the splitting of the α segment is observed (Fig. 3B). In Table II the values of the quadrupolar splittings of DOPC-d_4 are given for various L/P ratios. The T_{2e} relaxation time, which can be described by a single-exponential decay for all the systems measured, decreases with increasing protein content (Table I). For all samples the T_{1z} relaxation times of DOPC-d_4 are analyzed as a single-exponential decay and are independent of the L/P ratio (Table I).

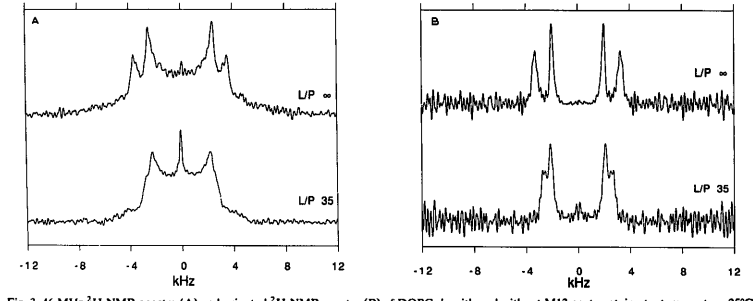

Fig. 3. 46 MHz ^{2}H-NMR spectra (A) and oriented ^{2}H-NMR spectra (B) of DOPC-d_4 with and without M13 coat protein at a temperature 25°C.

TABLE I

Relaxation times T_{1z} and T_{2e} for the on different positions deuterated phospholipids with and without the M13 coat protein

The used temperatures are 25°C for DOPC-d_4 and 40°C for DOPC-d_9. The L/P ratios are 35 for DOPC-d_4 and 40 for DOPC-d_9. The various DOPE systems with (L/P 38±4) and without M13 coat protein were prepared in lipid mixtures with the ratios; DOPE-d_4/PG and DOPE-d_4/PC (3:1, w/w) and DOPE-d_4/DOPG/CL (15:3:2, w/w/w) and measured at at 5°C. DOPG-d_5 with (L/P 38±4) and without M13 coat protein in the mixtures with DOPC and DOPE (3:1, w/w) were measured at 5°C. Samples were prepared and measured as described under Materials and Methods.

	T_{1z} (ms)		T_{2e} (ms)	
	pure	+M13	pure	+M13
DOPC-d_9	52 ±1.0	55 ±1.0	2.6 ±0.1	2.1 ±0.1
DOPC-d_4	8.9±1.0	8.4±1.0	0.60±0.05	0.40±0.05
DOPE-d_4/DOPC	5.9±0.1	7.0±0.1	0.97±0.05	0.55±0.05
DOPE-d_5/DOPG	6.9±0.1	7.0±0.1	0.86±0.05	0.78±0.05
DOPE-d_4/DOPG/CL	6.1±0.1	5.4±0.1	1.07±0.05	0.66±0.05
DOPG-d_5/DOPE	7.6±0.1	7.1±0.1	1.45±0.05	1.32±0.05
DOPG-d_5/DOPC	6.8±0.1	7.1±0.1	1.11±0.05	0.94±0.05

TABLE II

Quadrupolar splittings ($\Delta\nu_Q$ in kHz ± 0.05 kHz) of DOPC-d_4 with different M13 coat protein contents at 25°C

L/P	α	β
∞	6.70	4.10
55	6.00	4.30
35	5.60	4.40

Deuterium NMR spectra of bilayers consisting of the lipid mixtures DOPE-d_4/DOPC (3:1, w/w), DOPE-d_4/DOPG (3:1, w/w) or DOPE-d_4/DOPG/CL (15:3:2, w/w/w) are a superposition of the powder patterns from the α and β deuterons in the DOPE-d_4 headgroup (Fig. 1). The outer quadrupolar splitting is attributed to the α headgroup segment, the inner to the β headgroup segment, according to Seelig and Gally [25]. Deuterium NMR spectra of the lipid mixture DOPE-d_4/DOPG/CL with and without M13 coat protein are shown in Fig. 4, which demonstrate the decrease of the α quadrupolar splitting and the slight increase in the β splitting with increasing M13

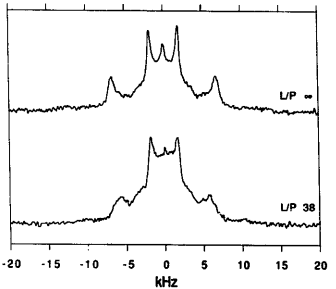

Fig. 4. 46 MHz ^{2}H-NMR spectra of DOPE-d_4/DOPG/CL (15:3:2, w/w) with and without M13 coat protein (L/P 38) at a temperature of 5°C.

coat protein content. This is a general feature for all the DOPE-d_4 systems as shown in Table III. In the lipid mixture DOPE-d_4/DOPC it is possible to distinguish both α deuterons, whereas this is not possible in bilayers of the lipid mixtures DOPE-d_4/DOPG and DOPE-d_4/DOPG/CL. The observation that the α-CD_2 of DOPE-d_4 gives rise to two quadrupolar splittings (Table III) has been shown previously in other systems [24,26,27]. In Table I the spin lattice relaxation times T_{1z} and the spin-spin relaxation times T_{2e} of DOPE-d_4 with different M13 coat protein content are given. There are no substantial changes in the T_{1z} relaxation times induced on adding M13 coat protein. In all lipid systems there is a significant decrease (up to 40%) of T_{2e} on adding M13 coat protein.

^{2}H-NMR spectra of the lipid mixture DOPE/DOPG-d_5 (3:1, w/w) bilayers with and without M13 coat protein shown in Fig. 5, are a superposition of the powder patterns from the α, β and γ headgroup deuterons. The outer quadrupolar splitting is attributed to the α headgroup segment, the next to the β headgroup segment and the inner quadrupolar splitting to the γ headgroup segment, according to Sixl and Watts [28]. Fig. 5 demonstrates the decrease in α, β and γ quadrupolar splittings induced by the incorpora-

TABLE III

Quadrupolar splitting ($\Delta\nu_Q$ in kHz ± 0.1 kHz (α) or ± 0.05 (β) kHz) of DOPE-d_4 with (L/P 38±4) and without M13 coat protein in DOPE-d_4/X (3:1, w/w) and DOPE-d_4/X/Y (15:3:2, w/w/w) systems at 5°C

	DOPC		DOPG		X = DOPG, Y = CL	
	−M13	+M13	−M13	+M13	−M13	+M13
α	12.8/12.2 [a]	11.1/10.5 [a]	13.0	12.8	13.5	12.7
β	3.70	3.90	3.90	4.30	4.00	4.10

[a] Two splittings are observed in the depaked spectra.

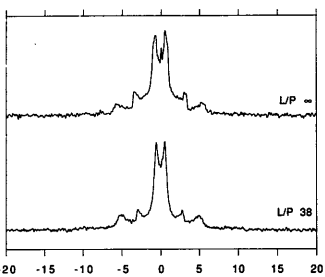

Fig. 5. 46 MHz ^{2}H-NMR spectra of DOPC/DOPG-d_5 (3:1, w/w) bilayers with and without M13 coat protein (L/P 38) at a temperature of 5°C.

tion of M13 coat protein. As is shown in Table IV, the effect of incorporation of M13 coat protein on the quadrupolar splittings of DOPG-d_5 is the same for both lipid mixtures DOPE/DOPG-d_5 and DOPC/DOPG-d_5 (3:1, w/w). The fact that more than one component is observed for the α and γ headgroup quadrupolar splittings in some cases is probably due the motional inequivalance, which could arise from the fact that DOPG-d_5 is in the headgroup racemic as a result of the synthetic procedure used for labeling [28,29]. In Table I the spin lattice relaxation times T_{1z} and the spin spin relaxation times T_{2e} of DOPG-d_5 with different M13 coat protein content are given. There is no substantial change in the T_{1z} relaxation times on adding M13 coat protein. The T_{2e} relaxation time decreases slightly (10–15%) in both systems on adding M13 coat protein.

The ^{31}P-NMR spectra (not shown) are typical for those obtained from bilayer systems with no indication of any second component. The chemical shift anisotropy (−36 ppm) of the spectra and the T_2 relaxation time (0.6 ± 0.1 ms, 17°C) of the ^{31}P nucleus of the DOPC bilayers are independent of the L/P ratio, within experimental error. Both in the pure lipid and samples containing protein there is an isotropic component observable in the ^{2}H and ^{31}P NMR spectra. This component is always less than 3% of the total intensity and is independent of the L/P ratio. This component is assigned to lipids in smaller vesicles, which rotate fast on the NMR time scale.

TABLE IV

Quadrupolar splitting ($\Delta\nu_Q$ in kHz ± 0.1 kHz (α) or ± 0.05 (β, γ) kHz) of DOPG-d_5 with (L/P 38 ± 4) and without M13 coat protein in X/DOPG-d_5 systems (3:1, w/w) at 5°C

	X = DOPG		X = DOPC	
	−M13	+M13	−M13	+M13
α	12.0	10.5	11.9/11.4 [a]	10.6
β	5.00	4.30	6.90	6.10
γ	1.10	0.90	1.90/1.40 [a]	1.60/1.20 [a]

[a] Two splittings are observed in the depaked spectra.

Discussion

The β-polymeric form of M13 coat protein has been investigated previously [1,9–11,13–15]. With NMR and ESR methods it has been shown that the protein in the β-polymeric form gives rise to a fraction of lipids trapped by the protein aggregates [1,12]. To test if this is also the case for the α-oligomeric form of the M13 coat protein we have performed ^{2}H-NMR measurements on DOPC-d_9. To study in more detail the interaction of the α-oligomeric form of M13 coat protein with the surrounding lipid matrix we have studied mixtures of the deuterated phospholipids DOPC-d_4, DOPE-d_4 and DOPG-d5, which mimic the *E. coli* membrane.

In ^{2}H-NMR spectra of DMPC-d_9 with M13 coat protein in the β-polymeric form two components were observed that were attributed to bulk and trapped lipids. Trapping lipids by lipid-protein complexes is possible, since the protein in the β-polymeric form is known to form large aggregates [1]. In contrast to the protein in the β-polymeric form, for the α-oligomeric form of M13 coat protein no trapped lipids are observed in the DOPC-d_9 ^{2}H-NMR spectra (Fig. 2). This is in agreement with the information obtained from HPLC, which show less strong aggregation for this form of the M13 coat protein. This means that in the case of the protein in the α-oligomeric form the spectrum is determined by a fast exchange of boundary lipids and lipids, which are not in contact with the protein. However, due to the fast exchange, it is not possible to obtain the amount of boundary lipids per protein subunit, because the quadrupolar splitting of the boundary lipids is unknown.

To study in more detail the interactions between M13 coat protein and phospholipids we have studied various lipid mixtures. Differences in the quadrupolar splitting of headgroup-labeled PC and PE for various mixtures of lipids have been reported previously [30]. The effect of M13 coat protein in the α-oligomeric form on both DOPC-d_4 as well as DOPE-d_4 is a decrease of the quadrupolar splitting associated with the α-methylene (CD_2) deuterons, whereas the quadrupolar splittings of the β-methylene (CD_2) deuterons show a corresponding increase (Table III). It has been shown with many other positively charged membrane substitutes that the effect observed with

DOPC-d_4 can be explained by a change in torsion angles within the headgroup as a result of the introduction of positive charges at the membrane surface [31,32]). Spectroscopic and crystallographic data suggest that the headgroup conformations for PC and PE are similar [33]. In addition the charge distribution of the PE and PC headgroups are alike. This explains the similar effect of M13 coat protein on the PE headgroup as compared to the effects on the PC headgroup.

Table IV demonstrates the decrease of the quadrupolar splittings of the glycerol headgroup, when M13 coat protein is introduced to the bilayers. In mixtures of DOPG/DOPE and DOPC/DOPE the same relative decrease for all splittings upon incorporation of M13 coat protein is observed. This suggests that a change in order induced by the protein is an effect of the whole glycerol moiety. The T_{1z} relaxation times of the deuterons in PG-headgroup and the phosphorus spectra are not influenced, therefore a change in the torsion angles α_3 and α_4 (Fig. 1C) can probably explain the changes of the quadrupolar splittings in the glycerol headgroup on addition of M13 coat protein. A decrease of all the quadrupolar splittings induced by adding a positively charged protein has also been observed previously on the introduction of myelin basic protein [34].

The differences between the observed changes in the quadrupolar splittings of headgroup deuterated PE and PC, as compared to the changes of the quadrupolar splittings of headgroup deuterated PG induced by the positive charges of the M13 coat protein, are probably due to the differences in hydrogen bonding capabilities and differences in charge distribution of the headgroups used. PE and PC have a net dipole, in contrast to PG, which has one negative charge. These differences result in a different conformation of the PE and PC headgroup as compared to the PG headgroup on introduction of positive charges at the membrane surface.

It can be concluded that in this system the changes induced by the M13 coat protein on the lipid headgroups may be due to a charge induced effect. However, the changes in quadrupolar splittings are small as compared to previous reported results upon introduction of positive amphiphiles in a lipid membrane [32]. This suggests a larger distance between the charges and the headgroup or a different a distribution of charges at the surface of the lipid bilayer in the case of the M13 coat protein as compared to the charges induced by the amphiphiles. Since the charge effect is small, only weak electrostatic interaction with the lipids occurs. Similar effects of positively charged M13 coat protein on DOPE-d_4 are observed in all the mixtures used, indeed showing no detectable specificity for the negatively charged lipids PG or CL.

The positive charges introduced by the protein at the membrane surface, causing the observed effects in the ^{2}H-NMR spectra, can be assigned to its positively charged lysine residues. It should be noted that lysine 40 is situated at the end of the hydrophobic part of the protein, therefore probably lying close to the membrane bilayer surface. Also lysine residues 43 and 44 are in the vicinity of the bilayer surface. Due to the dialysis procedure in preparing the lipid protein systems, the protein is probably randomly inserted in the membrane giving a completely symmetric bilayer with a net positive charge at both membrane surfaces.

The T_{2e} relaxation times of the α and β head-group labelled lipids DOPE-d_4 and DOPC-d_4 can be analysed as a single exponential decay. This indicates that both labeled headgroup segments of the phospholipid undergo similar motions. This is clearly not the case for the γ labels in comparison to the α and β labels of the lipids DOPG-d_5 and DOPC-d_4. This can also be observed in the spectra of DOPG-d_5 at different inter pulse delays (τ). After long delay times all the intensity of the signals originating from the α and β headgroup labels has disappeared, whereas the the quadrupolar splitting of the γ headgroup segment is still visible (results not shown). This means that the given relaxation time for DOPG-d_5 is a weighted average of the relaxation times of all labeled segments. The T_{2e} relaxation time of the γ segment deuterons of DOPG-d_5 can be estimated and is close to the relaxation times obtained for DOPC-d_9 whereas the relaxation time of the α and β-segment deuterons is close to the relaxation times obtained from the other lipids labelled at identical positions. This suggest that similar segments in the heagroups of different lipids undergo similar motions in lipid bilayers.

The value of T_{2e} decreases upon incorporation of M13 coat protein. This decrease of T_{2e} and the constant value of T_{1z} relaxation times of all systems investigated indicates that only slow motions are affected by M13 coat protein. A possibility to explain a decrease in the T_{2e} relaxation time upon incorporation of membrane proteins in lipid bilayers is that under intermediate exchange conditions the overall T_{2e} relaxation rate is the population average of boundary and bulk lipids plus an extra term, which takes into account the exchange process [35]. If it is assumed that the T_{2e} relaxation time for the lipid fractions influenced by the protein is identical to the relaxation time of the bulk lipids and the number of lipids in the boundary shell is at least four, this being the number of boundary lipids found in an aggregated system [1], then exchange rates are obtained, which would in the NMR spectra give rise to slow exchange. Since this is not the case, this would mean that the boundary lipids are undergoing a restricted motion as compared to the bulk lipids. This is in agreement with results obtained from ESR and time resolved fluorescence spectroscopy of M13 coat

protein incorporated in lipid bilayers (Sanders et al., in preperation).

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