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The Membrane Interaction of Amphiphilic Model Peptides Affects Phosphatidylserine Headgroup and Acyl Chain Order and Dynamics. Application of the "Phospholipid Headgroup Electrometer" Concept to Phosphatidylserine†

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Received July 5, 1990; Revised Manuscript Received September 28, 1990

ABSTRACT: Deuterium nuclear magnetic resonance (^{2}H NMR) was used to study the interaction of amphiphilic model peptides with model membranes consisting of 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine deuterated either at the β-position of the serine moiety ([2-^{2}H]DOPS) or at the 11-position of the acyl chains ([11,11-$^2H_2$]DOPS). The peptides are derived from the sequences H-Ala-Met-Leu-Trp-Ala-OH (AX, one-letter code with X = MLWA) and H-Arg-Met-Leu-Trp-Ala-OH (RX^+) and contain a positive charge of +1 ($AXme^+$) or +2 ($RXme^{2+}$) at the amino terminus or one positive charge at each end of the molecule ($AXetN^{2+}$). Upon titration of dispersions of DOPS with the peptides, the divalent peptides show a similar extent of binding to the DOPS bilayers, which is larger than that of the single charged peptide. Under these conditions the values of the quadrupolar splitting ($\Delta\nu_q$) of both [2-^{2}H]DOPS and [11,11-$^2H_2$]DOPS are decreased, indicating that the peptides reduce the order of both the DOPS headgroup and the acyl chains. The extent of the decrease depends on the amount of peptide bound and on the position of the charged moieties in the peptide molecule. The effects exerted by the peptides on the $\Delta\nu_q$ value of [2-^{2}H]DOPS are consistent with the PS headgroup responding as a molecular electrometer to the surface charge resulting from the presence of the peptides in the lipid–water interface. The effects on the acyl chain deuterons are in agreement with a localization of the peptides intercalated in between the lipid headgroups. Titrations of DOPS with poly(L-lysine)$_{100}$, which were included for reasons of comparison, reveal increased $\Delta\nu_q$ values. When the peptide–lipid titrations are carried out without applying a freeze–thaw procedure to achieve full equilibration, two-component ^{2}H NMR spectra occur. The apparently limited accessibility of the lipid to the peptides under these circumstances is discussed in relation to the ability of the peptides to exhibit transbilayer movement. ^{2}H spin–lattice relaxation time $T1$ measurements demonstrate a decrease of the rates of motion of both headgroup and acyl chains of DOPS in the presence of the peptides.

The interactions of peptides and proteins with the lipid phase of biological membranes are essential for many biological processes. Due to the complexity of biological membranes, model systems consisting of isolated compounds are often used to investigate at a molecular level how the mutual interaction of peptides and lipids may contribute to a particular biological function. A set of interrelated model peptides based on the sequence H-Ala-Met-Leu-Trp-Ala-OH has been designed for the purpose of studying molecular aspects of membrane insertion and translocation processes of peptides/proteins. In a previous study (de Kroon et al., 1990b) the lipid affinity and the membrane topology of several peptide derivatives varying in net charge and hydrophobicity have been assessed by using the fluorescence properties of the tryptophan residue. These peptides attain a localization in the membrane–water interface, the insertion depth depending on the peptides' charge and hydrophobicity. Particularly, the accessibility of the peptides' tryptophan residue to bromines attached at different positions along the phospholipids' acyl chains, reported in that study, raised questions concerning the ability of these peptides to perturb the acyl chains. Here the influence of these peptides on the order and dynamics of the lipid molecules at the level of the headgroup and the acyl chains is reported.

^{2}H NMR,[1] a nonperturbing biophysical technique, provides a suitable method to address these questions (Seelig & MacDonald, 1987). The main experimental parameters obtained from ^{2}H NMR encompass the deuterium quadrupole

† This work was supported by the Foundation for Biophysics with financial aid from the Netherlands Foundation for Scientific Research (NWO).
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[1] Abbreviations: NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine.

0006-2960/91/0430-1155$02.50/0

splitting ($\Delta\nu_q$), which provides information about the average orientation and the fluctuations of the C–^{2}H bond, and the deuterium relaxation times which characterize the dynamics of the system (Seelig, 1977). The present paper aims at characterizing the effects of several model peptides on the ^{2}H NMR parameters of phospholipid bilayers consisting of headgroup [β(2)-position of the serine moiety] and acyl chain (11-position) deuterated 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS).

Previous ^{2}H NMR studies have shown that integral membrane proteins in general have little effect on the deuteron order parameter of the phospholipid acyl chains [reviewed in Bloom and Smith (1985); Roux et al. (1989)]. Similar results have been found for the uncharged hydrophobic peptide gramicidin when incorporated into a bilayer (Chupin et al., 1987). Single positively charged tripeptides which are localized in the membrane–water interface did produce a significant reduction of lipid acyl chain order in perdeuterated DMPC bilayers (Jacobs & White, 1987). A strong decrease in lipid acyl chain order was observed upon binding of the mitochondrial precursor protein apocytochrome *c* to DOPS-containing bilayers (Jordi et al., 1990).

Changes in quadrupolar splitting of phospholipid headgroups are related to the net surface charge determined headgroup conformation rather than to the headgroup flexibility (Seelig et al., 1987). This was concluded from the counterdirectional change observed for the quadrupolar splittings of phosphatidylcholine, deuterated at the choline α- and β-position, in the presence of several lipophilic ions. The values of $\Delta\nu_q$ were found to vary linearly with the surface charge density (Akutsu & Seelig, 1981; Altenbach & Seelig, 1984; MacDonald & Seelig, 1988; Scherer & Seelig, 1989). The so-called "phospholipid headgroup electrometer" concept (Seelig et al., 1987) also applies to the interaction of polypeptides such as melittin and a cationic transmembrane modelpeptide with PC-containing model membranes (Kuchinka & Seelig, 1989; Dempsey et al., 1989; Beschiaschvili & Seelig, 1990; Roux et al., 1989). Recent studies involving acidic phospholipids indicate that a similar mechanism accounts for the variations of $\Delta\nu_q$'s of headgroup-deuterated DMPS in response to the cationic transmembrane polypeptide (Roux et al., 1989) and to melittin (Dempsey et al., 1989). In the case of melittin there may be an additional steric contribution by the peptide to the headgroup conformation (Dempsey et al., 1989).

The set of peptides used in the present study comprises the zwitterionic sequence H-Ala-Met-Leu-Trp-Ala-OH and three positively charged derivatives (de Kroon et al., 1990b). Methylation of the carboxyl terminus yielded a single positive charge at the N-terminus. The coupling of diaminoethane to the C-terminal Ala resulted in a peptide with a net charge of 2+ in which the positive charges are 5 amino acid residues apart. By substitution of the N-terminal Ala by an Arg residue, in addition to methylation of the C-terminus, a peptide with a net charge of 2+ residing on one amino acid was obtained. These peptides meet the requirements of showing binding to acidic phospholipids (de Kroon et al., 1990b) and of being buffer-soluble at the concentrations imposed by the NMR experiments.

By virtue of their high buffer solubility, these peptides could be directly titrated from stock solutions in DMSO to the DOPS dispersions. This enables the comparison of spectra, obtained in parallel titration experiments carried out with and without complete equilibrium of the lipid/peptide sample by a freeze–thaw procedure, on the basis of relative amounts of lipid accessible to the various peptides. As will be discussed, these data allow an interpretation in terms of different bilayer translocation abilities of the peptides.

The effects exerted by the peptides on the DOPS headgroup deuteron will be discussed within the frame of the phospholipid headgroup electrometer concept.

Materials and Methods

Peptides. The synthesis, modification and purification of the peptides have been described before (de Kroon et al., 1990b). The related peptides AMLWA (one-letter code, abbreviated as AX, with X designating the sequence MLWA) and RMLWA (RX^+) were synthesized, and derivatives were prepared by methylating the carboxyl group (Gattner et al., 1976) or by coupling diaminoethane to this moiety. Four peptides have been studied: the zwitterionic AX, its methylated analogue $AXme^+$, $RXme^{2+}$ with two positive charges on the arginine residue, and $AXetN^{2+}$ with one positive charge at each end of the molecule. The peptides were at least 97% pure as judged by analytical reverse-phase HPLC on Polyol-RP 18 (Serva, Heidelberg, FRG). Concentrations of peptide stock solutions in dimethyl sulfoxide (DMSO) were determined spectrophotometrically by using $\epsilon^{280} = 5600\ M^{-1}\ cm^{-1}$. Poly(L-lysine) hydrobromide with a polymerization degree of 100, $(Lys)_{100}$ (Sigma, St. Louis, MO), was added from stock solutions (prepared on weight basis) in deuterium-depleted buffer (see below). Amounts and concentrations of $(Lys)_{100}$ are always expressed per lysine monomer.

Phospholipids. The synthesis of 1,2-dioleoyl-*sn*-glycero-3-phospho-[2-^{2}H]-L-serine ([2-^{2}H]DOPS) has been reported previously (de Kroon et al., 1990a). 1,2-[11,11-$^2H_2$]-Dioleoyl-*sn*-3-glycerophospho-L-serine ([11,11-$^2H_2$]DOPS) was prepared by phospholipase D mediated base exchange from deuterated DOPC (Comfurius & Zwaal, 1977), which was synthesized as described [de Kroon et al. (1990a) and references cited therein]. Due to the synthesis procedure the 11,11-$^2H_2$-labeled dioleoyl phospholipids contain a minor amount of esterified [11,11-$^2H_2$]elaidic acid (6%; Chupin et al., 1987). Unlabeled DOPS was prepared according to standard procedures (van Deenen & de Haas, 1964; Comfurius & Zwaal, 1977).

Preparation of NMR Samples. DOPS lipid films (20 μmol of [2-^{2}H]DOPS, 10 μmol of [11,11-$^2H_2$]DOPS) were swollen for at least an hour in a buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.0, and 1 mM EDTA in deuterium-depleted water (Sigma) at a final lipid concentration of 30 mM. After 10 freeze–thaw cycles the pH was checked and adjusted if required to 7.2 ± 0.2 by adding HCl or NaOH (0.5 M in deuterium-depleted water) followed by another 10 freeze–thaw cycles. In the pH range 7.0–7.4 the quadrupolar splittings of both [2-^{2}H]DOPS and [11,11-$^2H_2$]DOPS do not change significantly (de Kroon et al., 1990a).

Titrations with the peptides were carried out by adding increasing amounts of the peptide stock solution to the lipid dispersion up to lipid/peptide ratios (mol/mol) of 100, 50, 25, 15, 10, 7.5, 5, 4, 3, and 2, respectively. Concentrations of stock solutions of the peptides in DMSO were such that in the titration experiments the final DMSO content of the NMR samples never exceeded 7% (v/v). At this concentration DMSO does not affect the ^{2}H NMR spectra of the lipid dispersions (data not shown). After each addition of peptide the sample was briefly agitated on a vortex mixer and, where indicated, subjected to 10 cycles of freeze–thawing to achieve complete equilibration. The pH was readjusted to 7.0–7.4 if necessary.

Peptide Binding Assay. The amount of peptide bound to the DOPS liposomes at each point of the peptide–lipid titration

up to a lipid/peptide molar ratio of 4 was assayed in separate experiments using nonlabeled DOPS. Aliquots (200 μL) of 30 mM DOPS were incubated for at least 2 h with the appropriate amounts of peptide, with or without 10 cycles of freeze–thawing. Half of each sample was centrifuged at 435000*g* for 60 min in a Beckman TL100 ultracentrifuge. The percentage of peptide bound was determined spectrophotometrically by measuring the absorbances at 280 nm of the supernatant and that of the not centrifuged half of the sample after addition of 0.5% (w/v) sodium cholate in order to solubilize the DOPS–peptide complexes. For the peptide AXetN^{2+}, in addition, 2 min of sonication in a bath sonicator was required to dissolve the DOPS–peptide aggregates that occurred above peptide/lipid ratios of 1/5. The amount of DOPS in the supernatants, determined according to Rouser et al. (1970), was negligible. Control experiments without lipid were performed which showed that up to 7.5 mM peptide (corresponding to a lipid/peptide ratio of 4) no AXme$^+$, RXme^{2+}, or AXetN^{2+} was spun down. Upon centrifugation of solutions of the zwitterionic peptide AX, already above a concentration of 2 mM, significant amounts of aggregated peptide (14% at 3 mM, 53% at 6 mM) were pelleted. For each molar ratio the lipid binding data were accordingly corrected for the amount of AX pelleted in the absence of lipid.

NMR. The NMR experiments were performed on a Bruker MSL-300 spectrometer at 18 °C. ^{2}H NMR spectra were acquired at 46.1 MHz by using the quadrupolar echo pulse sequence with a pulse length of 11.5 μs and a pulse separation of 35 μs. The interpulse times were 50 ms for [2-^{2}H]DOPS and 100 ms for [11,11-$^2H_2$]DOPS. The spectral width was 71.5 kHz, and an exponential filtering resulting in a 200-Hz line broadening was applied to the cumulative free induction decays. Spin–lattice relaxation times *T*1 were measured at different temperatures by employing an inversion–recovery sequence and were determined from a least-squares fit of the signal amplitudes. ^{31}P NMR spectra were recorded at 121 MHz by using a gated decoupling technique (Chupin et al., 1987), with a 14-μs 90° rf pulse, 38.5 kHz spectra width, and 1-s interpulse time. Exponential filtering of the free induction decays resulated in 50-Hz line broadening.

Fluorescence. The time dependence of the tryptophan fluorescence increase at 340 nm occurring upon the addition to the peptides of DOPS small unilamellar vesicles prepared by sonication as described (de Kroon et al., 1990b) was recorded on a SLM-Amino SPF-500C fluorimeter. The excitation wavelength was 280 nm, and the temperature was kept at 25 °C. To a 6 μM peptide solution in buffer which was continuously stirred was added DOPS to a concentration at which the binding of the peptide is incomplete (Jain et al., 1985a,b). The fluorescence traces were corrected for a vesicle blank and normalized to a trace recorded for the amino acid tryptophan under identical conditions in order to correct for the inner-filter effect (de Kroon et al., 1990b).

Results

Aqueous dispersions of the deuterated DOPS at neutral pH are organized in bilayers and give rise to axially symmetric ^{2}H NMR spectra with single characteristic quadrupole splittings of 14.2 and 7.3 kHz, respectively, for [2-^{2}H]DOPS and [11,11-$^2H_2$]DOPS at 20 °C (de Kroon et al., 1990a). Figure 1 shows a set of typical ^{2}H NMR spectra of [2-^{2}H]-DOPS and [11,11-$^2H_2$]DOPS which are encountered upon titration with the peptides. The addition of the peptide RXme^{2+} to the lipid dispersion gives rise to two spectral components (Figure 1a,c), one with a $\Delta\nu_q$ value corresponding to that of the pure lipid and the other with a reduced $\Delta\nu_q$ value

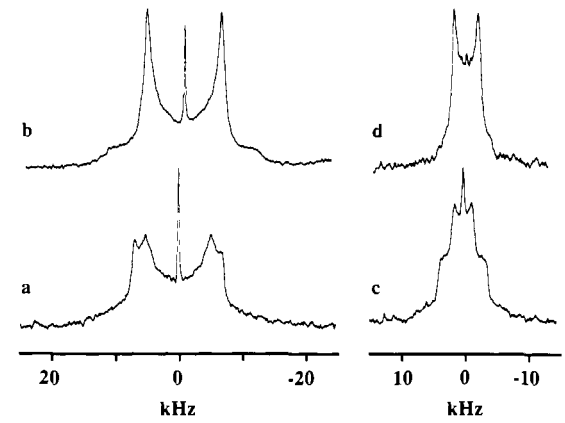

FIGURE 1: ^{2}H NMR spectra of [2-^{2}H]DOPS (a, b) and [11,11-$^2H_2$]DOPS (c, d) in the presence of RXme^{2+} at a molar ratio of 1 peptide added per 5 DOPS molecules before (a, c) and after 10 cycles of freeze–thawing (b, d).

depending on the amount of peptide added. These data indicate that only part of the lipid is accessible to the externally added peptide RXme^{2+}. Complete accessibility of the lipid to the peptides is achieved upon equilibrating the sample by a freeze–thaw procedure as evidenced by the ^{2}H NMR spectra with single $\Delta\nu_q$ values which are smaller than those of the pure lipids (Figure 1b,d). The other amphiphilic peptides also induce a decrease of the $\Delta\nu_q$ value of headgroup-deuterated DOPS (see below). $(Lys)_{100}$ affects the $\Delta\nu_q$ of [2-^{2}H]DOPS in the opposite direction: it gradually increases with increasing amounts of $(Lys)_{100}$ added to a maximum value of 16 kHz at a molar ratio $(Lys)_{100}$/DOPS of 2 (not shown). Concomitantly with the changes in $\Delta\nu_q$ the peptides and (Lys)100 give rise to an increased line width of the spectra (not shown). Nevertheless, reliable $\Delta\nu_q$ values can be measured even at a peptide added/lipid ratio of 1/2.

^{31}P NMR characterization of the samples, carried out in parallel, reveals axially symmetric powder spectra, which are typical for lipids organized in extended bilayers with no contributions from isotropic phases (data not shown). At the higher peptide/lipid ratios again some line broading is apparent but the chemical shift anisotropic (CSA) remains constant at ~53 ppm [cf. Browning and Seelig (1980)] up to peptide/DOPS ratios of 1/4. Above this ratio the peptide AXetN^{2+} causes massive line broadening and an increased CSA of ~60 ppm but the bilayer configuration is maintained (data not shown). At these high peptide/lipid ratios a loss of signal intensity is apparent in both the ^{2}H and ^{31}P NMR spectra.

In order to quantitatively compare the effects of the different peptides on the quadrupolar splittings of the deuterated DOPS, the binding of the peptides to DOPS liposomes under the NMR conditions was determined by a centrifugation assay. At the highest peptide concentration used in this assay (7.5 mM) the charged peptides are still completely soluble in buffer whereas the zwitterionic peptide starts to precipitate above 2 mM (see Materials and Methods). The affinity for DOPS was found to depend on the peptides' net charge. As is shown in Figure 2, the divalent positively charged peptides RXme^{2+} and AXetN^{2+} show nearly quantitative binding to DOPS liposomes in the concentration range of interest. The binding efficiency of the singly charged peptide AXme$^+$ is somewhat less while that of the zwitterionic AX is very low (Figure 2). Omission of the freeze–thaw procedure in the binding experiments decreases the binding efficiencies by at most 10% of the values obtained for the completely equilibrated samples in the range of 0–0.25 mol of peptide added per mole of DOPS (data not shown).

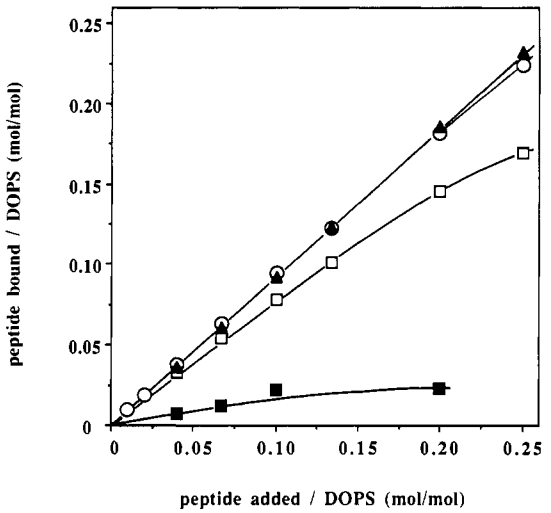

FIGURE 2: Amounts of peptide bound to DOPS liposomes plotted against the amounts of peptide added for AX (■), $AXme^+$ (□), $RXme^{2+}$ (○), and $AXetN^{2+}$ (▲). Samples were freeze–thawed 10 times prior to centrifugation; the DOPS concentration was 30 mM.

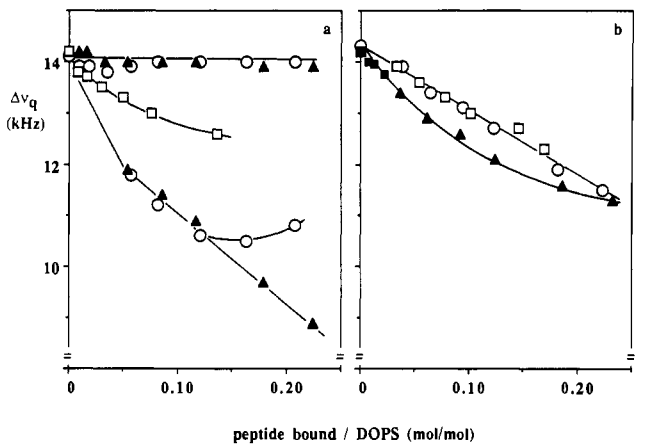

FIGURE 3: Variation of the quadrupolar splitting ($\Delta\nu_q$) of [2-^{2}H]DOPS in peptide titration experiments without (a) and with 10 cycles of freeze–thawing after each addition of peptide (b), plotted against the amount of peptide bound per DOPS, for the peptides $AXme^+$ (□), $RXme^{2+}$ (○), $AXetN^{2+}$ (▲), and AX (■); the latter was not included in the titration without freeze–thawing.

In Figure 3 the $\Delta\nu_q$ values of [2-^{2}H]DOPS obtained in titrations with and without sample equilibrium are plotted against the amount of peptide bound. When the freeze–thaw procedure during the titrations with $RXme^{2+}$ and $AXetN^{2+}$ is omitted, two spectral components are readily discerned, one with a constant $\Delta\nu_q$ value characteristic for the pure lipid and the other one changing in response to the peptide (Figure 1a and 3a). The relative signal intensities of the two spectral components at a certain peptide/lipid ratio are stable over longer periods of time (at least 24 h). In the titration of [2-^{2}H]DOPS with $AXetN^{2+}$ the $\Delta\nu_q$ of the second component decreases in a linear fashion with the amount of peptide bound whereas for $RXme^{2+}$ the decrease is reversed above a peptide bound/DOPS molar ratio of 0.11 (Figure 3a). The titration of [2-^{2}H]DOPS with $AXme^+$ does not yield two resolved spectral components. However, from the difference observed for the $\Delta\nu_q$ decreases in response to this peptide without and with freeze–thawing (Figure 3, panels a and b) it can be concluded that at least at low peptide/lipid ratios not all of the lipid is affected by the externally added $AXme^+$.

All ^{2}H NMR spectra of samples equilibrated by freeze–thawing exhibit a single quadrupolar splitting (Figure 3b), indicating that the residence time of a peptide molecule at a particular DOPS molecule is short on the ^{2}H NMR time scale ($\sim 10^{-5}$ s). The peptides $AXme^+$ and $RXme^{2+}$ equally affect the $\Delta\nu_q$ of the headgroup deuteron under conditions of sample equilibration. The decrease of $\Delta\nu_q$ induced by these peptides depends linearly on the amount of peptide bound (Figure 3b). In contrast, for the peptide with a charge at each end of the molecule, $AXetN^{2+}$, such a linear relation is not observed. This peptide is more efficient in reducing the headgroup $\Delta\nu_q$ than the others, but the efficiency decreases as more peptide is bound.

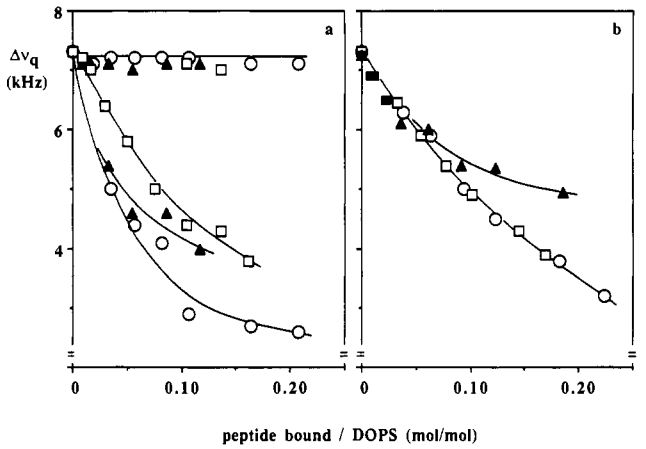

FIGURE 4: Peptide titrations of [11,11-$^2H_2$]DOPS; panels a and b and the symbols as in Figure 3. Due to the strong acyl chain disordering effect of $AXetN^{2+}$ no defined $\Delta\nu_q$ values could be obtained above a molar ratio of $AXetN^{2+}$ bound/DOPS of 0.12 (panel a).

The effect of the peptides on the DOPS acyl chains was studied in parallel experiments using [11,11-$^2H_2$]DOPS (Figure 4). The divalent peptides $AXetN^{2+}$ and $RXme^{2+}$ which share a similar affinity for DOPS (Figure 2) affect the [11,11-$^2H_2$]DOPS spectra in different ways. While the reduction of $\Delta\nu_q$ induced by the peptide with the charges at one end of the molecule is larger than that induced by the peptide with a charge at each end (Figure 4b), the extent of line broadening caused by the latter is much greater (not shown). Figure 4b shows that $AXme^+$ and $RXme^{2+}$ are equally effective in decreasing the $\Delta\nu_q$ value of [11,11-$^2H_2$]DOPS per molecule bound, as was found for [2-^{2}H]DOPS, but this time the decrease is not linear with the amount of peptide bound (cf. Figure 3b and 4b). It may also be noted that the relative changes of $\Delta\nu_q$ in response to the peptides are larger for the acyl chain than for the headgroup-deuterated DOPS. Addition of $(Lys)_{100}$ to [11,11-$^2H_2$]DOPS results in a slight increase of $\Delta\nu_q$ to 8.0 kHz at a 2/1 $(Lys)_{100}$/DOPS molar ratio (not shown). The effect on $\Delta\nu_q$, per peptide bound, of the zwitterionic peptide AX, which has a small binding affinity for DOPS (Figure 2), qualitatively agrees with that of the other peptides for both [2-^{2}H]DOPS and [11,11-$^2H_2$]DOPS (Figure 3b and 4b).

With respect to the occurrence of two-component spectra in the nonequilibrated samples the response of [11,11-$^2H_2$]-DOPS is similar to that of [2-^{2}H]DOPS (cf. Figures 1, 3a, and 4a). In a titration of [11,11-$^2H_2$]DOPS with the peptide $AXme^+$ the appearance of a spectral component of low intensity with the original $\Delta\nu_q$, which is estimated to represent at most 10% of the total lipid (data not shown), is confined to the range of 0.1–0.15 peptide/DOPS bound (Figure 4a). At higher molar ratios it disappears (not shown). At lower molar ratios as well as in the parallel titration of [2-^{2}H]DOPS (see above) this low-intensity component cannot be discerned probably because of overlap by the high-intensity spectral component, for which the $\Delta\nu_q$ value is still too large to allow

Table I: Effect of the Peptides and $(Lys)_{100}$ on the 46.1-MHz ^{2}H $T1$ Relaxation Times at 30 °C of [11,11-$^2H_2$]DOPS

peptide[a]	$T1$ (ms)[b]	peptide[a]	$T1$ (ms)[b]
	24	$AXetN^{2+}$	16
$AXme^+$	20	$(Lys)_{100}$	25
$RXme^{2+}$	19		

[a] Peptides were added to a 1/2 and $(Lys)_{100}$ to a 2/1 molar ratio with respect to DOPS; samples were freeze–thawed 10 times. [b] The maximum error in the $T1$ values is 1 ms.

resolution of both components.

Deuterium $T1$ relaxation times have been measured in order to investigate the influence of the interacting peptides on the motional properties of the acyl chains and headgroup of DOPS. Table I shows that the ^{2}H $T1$ value of [11,11-$^2H_2$]DOPS is decreased upon addition of 0.5 mol of peptide/mol of DOPS. Since the $T1$ values increase with increasing temperatures and since the Arrhenius representations of the $T1$ data (ln $T1$ vs $1/T$, not shown) yield straight lines, the motions determining $T1$ fall in the fast correlation time regime. Therefore, the observed decreases in $T1$ are interpreted as decreases in the rate of acyl chain motion. No attempts have been made to compare the effects on $T1$ by the different peptides per peptide bound. The presence of a 2-fold molar excess of $(Lys)_{100}$ over DOPS does not significantly affect the $T1$ value of the acyl chain deuterons (Table I).

The DOPS headgroup deuteron exhibits very short $T1$ values (~7.5 ms at 30 °C) to which the fast correlation time limit applies (de Kroon et al,. 1990a). With $(Lys)_{100}$ present at a 2/1 molar ratio with respect to [2-^{2}H]DOPS, the $T1$ values above 20 °C are considerably decreased (e.g., to 5 ms at 30 °C) and a broad ^{2}H $T1$ minimum occurs at ~20 °C (data not shown). Due to the decreased signal/noise ratio at high peptide concentrations it was not possible to obtain a conclusive temperature dependence of the headgroup $T1$ in the presence of the amphiphilic peptides. In the temperature range above 20 °C the peptides cause a reduction of the $T1$ values comparable to $(Lys)_{100}$ (data not shown).

Discussion

In this study the effect of model peptides on the ^{2}H NMR characteristics of DOPS model membranes has been investigated in a comparative way. The main variables studies were the peptides' net charge and, for the divalent peptides, the charge distribution in the peptide. The affinity of these peptides for negatively charged bilayers is largely determined by electrostatic attraction with some contribution from hydrophobic interactions (de Kroon et al., 1990b). This is reflected in the stronger binding of the 2+ charged peptides as compared to the peptide carrying a single positive charge (Figure 2), in agreement with earlier results (de Kroon et al., 1990b). Throughout the titration experiments up to peptide/DOPS ratios of 1/2 the bilayer configuration of DOPS was maintained as evidenced by the ^{31}P NMR measurements. Above peptide/lipid ratios of 1/5 the formation of aggregated complexes was visible, most conspicuously in the case of $AXetN^{2+}$.

The changes of the ^{2}H quadrupolar splittings ($\Delta\nu_q$) of the [2-^{2}H]- and [11,11-$^2H_2$]DOPS in response to titration with the peptides depend on the amount of peptide bound and the nature of the peptide. The quadrupolar splitting provides a direct measure of the segmental order of the C–^{2}H bond according to

$$\Delta\nu_q = \tfrac{3}{4}\left(\frac{e^2qQ}{h}\right)S_{CD}$$

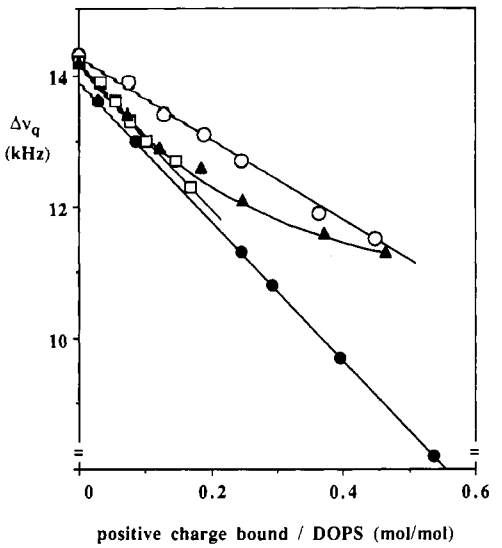

FIGURE 5: Variation of the quadrupolar splitting ($\Delta\nu_q$) of [2-^{2}H]DOPS with the amount of positive charges bound per mole of DOPS. The $\Delta\nu_q$ values originate from titrations with $AXme^+$ (□), $RXme^{2+}$ (○), and $AXetN^{2+}$ (▲) and were measured at 18 °C after freeze–thawing (see legend to Figure 3b). For comparison the $\Delta\nu_q$ values obtained in a pH titration of [2-^{2}H]DOPS with HCl (●) carried out at 20 °C (de Kroon et al., 1990a) were included, plotted against the protonation degree of DOPS (see text).

(where e^2qQ/h is the static quadrupole coupling constant, which is 170 kHz for an aliphatic C–^{2}H bond, and S_{CD} is the order parameter; Seelig, 1977). Both the average orientation of the C–^{2}H bond with respect to the axis of motional averaging (the bilayer normal) and the amplitude of the motions that are fast on the ^{2}H NMR time scale and motionally average the anisotropy of the membrane system determine the value of $\Delta\nu_q$ (Davis, 1983).

The main observation made in this study is that the amphiphilic peptides reduce the $\Delta\nu_q$ values and thus the order parameter of both the DOPS headgroup and the acyl chains. An obvious explanation for these concerted $\Delta\nu_q$ decreases would be that the inserted peptides decrease the order of the entire DOPS molecule. While we cannot rigorously exclude this possibility, we will outline below that two different and probably independent mechanisms are likely to account for the peptide-induced $\Delta\nu_q$ decreases: the $\Delta\nu_q$ value of the headgroup deuteron responds to the headgroup orientation which is determined by the membrane surface charge whereas the $\Delta\nu_q$ value of the acyl chain deuterons reflects the amplitude of the C–^{2}H bond angular fluctuations.

Headgroup-Deuterated DOPS. Roux et al. (1989) have shown that the quadrupolar splittings of the β (2-position) and α_1 (1-position) deuteron in the DMPS headgroup linearly decrease whereas that of the α_2 deuteron is not affected upon interaction of the lipid with increasing amounts of a cationic transmembrane peptide. They concluded that the magnitude of these $\Delta\nu_q$ values depends on the average orientation of the headgroup which responds to changes in surface charge induced by the binding of charged amphiphiles, rather than on the amplitude of the C–^{2}H bond angular fluctuations, in analogy to the PC headgroup for which the electrometer concept was proposed (Seelig et al., 1987). Recently, similar results have been obtained for headgroup-deuterated POPS (Roux & Bloom, 1990).

Here a different approach is presented to demonstrate that the peptide-induced changes in the $\Delta\nu_q$ of [2-^{2}H]DOPS reflect changes in surface charge according to the electrometer concept. Figure 5 compares the dependence of $\Delta\nu_q$ on the amount

of positive charges bound per mole of DOPS for the peptides (data taken from Figure 3b) to its dependence on the protonation degree of DOPS. Since the local pH at the PS surface is some 3 pH units lower than the bulk pH (MacDonald et al., 1976), the vast majority of the peptide molecules will be completely protonated when membrane bound, which justifies the use of net charges 1+ and 2+ for the peptides. The pH dependence of the $\Delta\nu_q$ of [2-^{2}H]DOPS (de Kroon et al., 1990a) has been converted into the protonation degree dependence by using an apparent pK_a of the PS carboxyl group of 4.5 (MacDonald et al., 1976; Hauser & Phillips, 1979; Boggs, 1987).

The protonation degree dependent linear decrease of $\Delta\nu_q$ is virtually identical with that observed in response to the binding of AXme$^+$, the small divergence being due to the 2 °C temperature difference between the experiments. This shows that AXme$^+$ exerts its effect on the [2-^{2}H]DOPS $\Delta\nu_q$ simply by neutralizing part of the negative surface charge and that there is no additional effect on the headgroup flexibility, as expected on the basis of the electrometer concept.

Next we will discuss how the [2-^{2}H]DOPS response to the divalent peptides fits into the electrometer concept. RXme^{2+} and AXme$^+$ give rise to identical linear $\Delta\nu_q$ dependences on the molar amount of peptide bound (Figure 3b), which indicates that the effective charge of RXme^{2+} experienced by the PS headgroups is only 1+. This result is consistent with the extended Gouy–Chapman theory that takes into account the finite size of divalent cations, from which it is predicted that when the distance of the charges on a divalent molecule is comparable to the Debye length, the effective charge felt at the membrane surface is decreased (Carnie & McLaughlin, 1983; Alvarez et al., 1983). The distance between the charges of the α-amino group and the side-chain guanidinium group in the N-terminal arginine residue is approximately 9 Å (using average C–C and C–N bond lengths of 1.5 Å and 120° bond angles) and almost equals the Debye length in 100 mM NaCl of 10 Å. This provides an obvious explanation for the effective electric charge of RXme^{2+} felt at the membrane surface being only half of its true electric charge. Accordingly, the effective charge of melittin as derived from experiments employing headgroup-deuterated POPC was determined to be 2.2 instead of its true electric charge of 6+ (Kuchinka & Seelig, 1989). Interestingly, it is in particular the two arginine residues in melittin that contribute little to the interfacial charge (Stankowski & Schwarz, 1990).

In the other divalent peptide AXetN^{2+} the distance between the charged groups is estimated to be ~30 Å, assuming an extended conformation. The interpretation of the $\Delta\nu_q$ change in response to this peptide is more complicated. At low amounts of AXetN^{2+} bound per DOPS the decrease in $\Delta\nu_q$ per charge bound parallels that of AXme$^+$ (Figure 5), suggesting that under these conditions both charges on the peptide independently interact with the membrane. At ratios above 0.2 positive charge bound per DOPS, a deflection from linearity sets in and the curve gradually approaches the line obtained for RXme^{2+} (Figure 5). Two possible explanations for this deflection are as follows: (1) Due to steric hindrance at higher peptide/lipid ratios AXetN^{2+} can only bind with one end to the bilayer surface. (2) Upon additional AXetN^{2+} binding above a positive charge/DOPS ratio of 0.2 the inter positive charge distance at the DOPS surface becomes comparable to the Debye length, thus reducing the effective positive charge as discussed above. Since the molecular dimensions of the peptide in a membrane-bound configuration are unknown, this point will not be pursued here.

In agreement with earlier results obtained with pentalysine (Roux et al., 1988) and in contrast to the amphiphilic peptides, $(Lys)_{100}$ causes an increase in the $\Delta\nu_q$ of [2-^{2}H]DOPS. This is thought to be due to the localization of the charged Lys residues at the external side of the headgroup region where they affect the headgroup orientation in the opposite direction (Roux et al., 1989).

Acyl Chain Deuterated DOPS. With increasing amounts of peptide bound the $\Delta\nu_q$ of the DOPS acyl chain deuterons is decreased, indicative of an increased motional freedom of the acyl chains. The two peptides AXme$^+$ and RXme^{2+} with the positive charge(s) residing at one end of the molecule induce an identical decrease of $\Delta\nu_q$ to ~50% of the value of the pure lipid, at a molar ratio of peptide bound per DOPS of 0.2 (Figure 4b). For the peptide with a positive charge at each end of the molecule, AXetN^{2+}, the decrease of $\Delta\nu_q$ is less. This difference probably reflects the different modes of anchoring of the peptides at the membrane surface, either by one or by two ends of the molecule, which affects the mode of insertion and consequently the extent of acyl chain perturbation. A similar difference is apparent in the exposure of the peptides' tryptophan residues to acyl chain brominated phosphatidylcholines incorporated in cardiolipin vesicles. Although the extent of association of the peptides RXme^{2+} and AXetN^{2+} with these vesicles is similar, the tryptophan fluorescence efficiency of quenching by the bromines of RXme^{2+} is twice that of AXetN^{2+} (de Kroon et al., 1990b). Unlike AXme$^+$ and RXme^{2+}, AXetN^{2+} causes considerable line broadening of the [11,11-$^2H_2$]DOPS spectra (not shown), indicative of a substantial spread in the average local order of the lipid acyl chains, for which also the surface anchoring by two ends of AXetN^{2+} is held responsible.

The observation that poly(L-lysine) bound to DOPS has a small acyl chain ordering effect is consistent with the notion that this molecule only interacts electrostatically at the bilayer surface and does not penetrate into the apolar part (de Kruijff et al., 1985; Roux et al., 1989). The cross-linking of the PS headgroups by $(Lys)_{100}$ results in a closer packing of the acyl chains. Upon reduction of the electrostatic repulsion of the PS headgroups by lowering the pH, a similar increase of the $\Delta\nu_q$ value of [11,11-$^2H_2$]DOPS is observed (de Kroon et al., 1990a). It should be emphasized that under these conditions the $\Delta\nu_q$ of the headgroup changes in the opposite direction: it displays a surface charge dependent decrease of $\Delta\nu_q$ (see above). This result reinforces the notion of two independent mechanisms acting on the quadrupole splittings of headgroup and acyl chains. In the case of the binding of positively charged amphiphilic peptides to DOPS the partial neutralization of the DOPS surface charge is not reflected in an increased $\Delta\nu_q$ of the acyl chains, since the increased order to be expected on the basis of a reduced electrostatic headgroup repulsion alone is amply exceeded by the opposite effect of the increased motional freedom of the acyl chains induced by the inserted peptides.

The property of disturbing the phospholipid acyl chain order appears to be confined to peptides/proteins which attain a localization in the lipid–water interface [cf. Jacobs and White (1987, 1989)]. The influence on acyl chain structure of integral membrane proteins and of a completely membrane-incorporated peptide like gramicidin is much less (see the introduction). The same goes for superficially membrane-adsorbed peptides and proteins like, e.g., polylysine and cytochrome *c* (Devaux et al., 1986). This suggests that the reduction in acyl chain order is the result of peptide-induced spacing of the lipid molecules at the level of the lipid head-

groups. Since membrane incorporation of a transmembrane peptide like $K_2GL_{20}K_2A$ (Roux et al., 1989) would be expected to cause a similar headgroup spacing, here the absence of an effect on acyl chain order is probably due to the transmembrane part of the peptide preventing an increased motional freedom. On the other hand, the mitochondrial precursor protein apocytochrome *c* which is thought to partially penetrate into the bilayer shows similar effects on the DOPS acyl chain order as the model peptides used in this study (Jordi et al., 1990). In fact, a quantitative comparison of $\Delta\nu_q$ values based on the amount of positive charges bound per DOPS molecule reveals fully comparable decreases upon titration with apocytochrome *c* (104 amino acids with a net charge of 9+) or, e.g., $RXme^{2+}$ (5 amino acids and 2+).

The conclusion can be drawn that perturbation of the DOPS acyl chains at the 11-position is a general property of peptides/proteins that intercalate in between the PS headgroups. In this context it should be mentioned that at the 9,10-position of POPC membranes only small changes in acyl chain order are observed upon interaction with melittin (Kuchinka & Seelig, 1989).

Dynamics. The ^{2}H $T1$ spin–lattice relaxation time measurements provide information about the changes in rate of molecular motions of DOPS in response to interaction with the peptides. The acyl chain deuterons display a limited peptide-induced decrease in the rate of motions determining $T1$. $(Lys)_{100}$, in contrast, does not affect the rate of acyl chain motions, reinforcing the different modes of binding of the amphiphilic peptides and $(Lys)_{100}$.

The $T1$ of the headgroup deuteron is also affected by the peptides. The PS headgroup which is already known for its small $T1$ values resulting from its slow rate of reorientation due to a rigid bilayer packing (Browning & Seelig, 1980; Browning, 1981; de Kroon et al., 1990a) appears to be even more immobilized upon binding of the peptides or $(Lys)_{100}$. The $(Lys)_{100}$/DOPS sample revealed a $T1$ minimum around 20 °C (not shown). This $T1$ minimum allows the unambiguous assessment of the correlation time, τ_c, of which the value suggests that the rate of the molecular motions of the PS headgroup determining $T1$ is considerably slowed down by the electrostatic interaction with $(Lys)_{100}$ (de Kroon et al., 1990a). Although no distinct $T1$ minima could be discerned in the peptide-containing samples, the peptides seem to affect the headgroup motions in a similar fashion. A decrease in $T1$ appears to be a general feature of phospholipid headgroup deuterons upon interaction with peptides or proteins [see, e.g., Tamm and Seelig (1983), Sixl et al. (1984), Sixl and Watts (1985), and Devaux et al. (1986)].

Lipid Accessibility and Peptide Translocation. Finally, the different lipid accessibilities to the peptides when added externally will be discussed. While the peptide $AXme^+$ affects most of the lipid molecules, the divalent peptides $RXme^{2+}$ and $AXetN^{2+}$ only interact with part of the lipid present. This is evident from a comparison of the data from the completely equilibrated samples to those obtained when the freeze–thaw procedure was omitted (Figures 3 and 4). The ratio of the number of DOPS molecules with a reduced $\Delta\nu_q$ over that with the original $\Delta\nu_q$ is much higher for the monovalent peptide than for the divalent ones (data not shown). The [2-^{2}H]DOPS titration with $AXetN^{2+}$ (Figure 3a) shows a linear relation between the $\Delta\nu_q$ value of the affected spectral component and the amount of peptide bound in the peptide bound/DOPS range of 0.05–0.25 (mol/mol). Extrapolating from Figure 3b that at the membrane surface density of $AXetN^{2+}$ in this range of peptide bound per lipid (corresponding to $\Delta\nu_q$ values below 12 kHz) the $\Delta\nu_q$ will decrease linearly with the amount of $AXetN^{2+}$ bound, this result would indicate that $AXetN^{2+}$ only binds to a fixed subpopulation of the lipid. From the slopes of the $\Delta\nu_q$ decreases in Figure 3 this subpopulation is estimated to be ~65% of the total lipid, which is somewhat larger than the amount of lipid expected to be present in the outer leaflet of the DOPS liposomes. Previous work has shown that upon hydration of DOPS at neutral pH predominantly large unilamellar vesicles are formed (Hauser, 1984). The other divalent peptide $RXme^{2+}$ gives rise to similar two-component spectra as $AXetN^{2+}$ up to a peptide/DOPS ratio of 0.11 (Figure 3a). For this peptide a linear relation between $\Delta\nu_q$ and the amount of peptide bound per lipid has been demonstrated (Figure 3b). Therefore, the extrapolation made above for $AXetN^{2+}$ seems valid.

Consequently, the limited lipid accessibility of the divalent peptides as compared to the monovalent one is interpreted as a lack of permeation, i.e., translocation by diffusion, of the divalent peptides across the DOPS bilayers, leaving the lipid molecules of the inner membrane leaflet unaffected. However, at peptide/DOPS molar ratios greater than 0.11, $RXme^{2+}$ apparently is able to gain access to the inner membrane leaflet, as evidenced by the increasing $\Delta\nu_q$ value of the affected spectral component above this ratio (Figure 3a). This translocation of $RXme^{2+}$ probably occurs via a mechanism in which this peptide bound in high concentrations to the DOPS bilayers is able to collapse the integrity of the vesicles, rendering all lipid molecules accessible to the peptide. $AXetN^{2+}$ does not show this ability in the concentration range studied due to its different mode of membrane anchoring (see above).

From the biphasic kinetics of the tryptophan fluorescence increase observed upon addition of PC vesicles to comparable neutral or single-charged model peptides, under conditions where not all of the peptide is bound, Jain et al. (1985a,b) have implied that these peptides exhibit transbilayer movement. They observed an instantaneous fluorescence increase within 10 s after adding the vesicles, which was followed by a slower increase with a half-time of several minutes. The fast increase is thought to represent insertion of the peptides into the outer leaflet of the membrane and the second phase a gradual transbilayer movement of the peptide from the outer to the inner leaflet. In order to examine the occurrence of transbilayer movement according to this criterion, similar fluorescence measurements have been applied to the peptides upon addition of DOPS. Because of the inner-filter effect and the large scatter contribution inherently attached to fluorescence measurements with DOPS dispersions as used in the NMR experiments, the results obtained with small unilamellar DOPS vesicles are depicted (Figure 6). Experiments employing the DOPS dispersions yielded qualitatively similar results (data not shown). The relative fluorescence increase (F/F_0) is larger for the divalent peptides than for $AXme^+$, in agreement with the results obtained with cardiolipin vesicles (de Kroon et al., 1990b). The kinetics of the fluorescence increases are different: after the immediate fluorescence increase within 10 s after adding the vesicles observed for each of the three peptides, $AXme^+$ clearly shows a gradual second-phase fluorescence increase, whereas the fluorescence intensity of the divalent peptides remains at the initially attained level (Figure 6). Following the interpretation of Jain et al. (1985a,b) this result corroborates our conclusion from the ^{2}H NMR data that in contrast to $AXme^+$ the divalent peptides do not equilibrate across the DOPS bilayer. The data are consistent with a mechanism in which the peptides are membrane permeable in the deprotonated form, carrying no

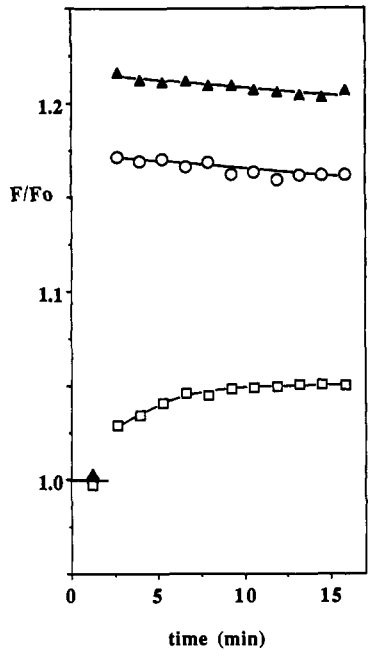

FIGURE 6: Time dependence of the tryptophan fluorescence emission quantum yield at 340 nm related to the initial fluorescence level F_0 upon addition of DOPS small unilamellar vesicles to the peptides $AXme^+$ (□), $RXme^{2+}$ (○), and $AXetN^{2+}$ (▲) at $t = 2$ min. The peptide concentration was 6 μM, and DOPS was added to a lipid/peptide molar ratio of 15 (○, ▲) or 30 (□).

charges. Experiments in which the pH-gradient-dependent uptake of these peptides into liposomes is studied corroborate such a mechanism (de Kroon et al., unpublished results).

ACKNOWLEDGMENTS

We thank Mrs. Gerda de Korte-Kool for purifying most of the peptides used in this study.

Registry No. AMLWA, 122149-26-2; $AXme^+$, 122130-24-9; $RXme^+$, 128660-82-2; $AXetN^{2+}$, 128660-81-1; DOPS, 70614-14-1; poly(L-lysine) SRU, 38000-06-5; poly(L-lysine) homopolymer, 25104-18-1.

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