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Interactions of an antimicrobial peptide, tachyplesin I, with lipid membranes

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Tachyplesin I, isolated from the acid extracts of hemocytes of *Tachypleus tridentatus*, is a cyclic broad-spectrum antimicrobial peptide forming a rigid, antiparallel β-sheet because of two intramolecular S-S linkages. The strong binding of the peptide to lipopolysaccharides cannot explain the susceptibilities of gram positive bacteria and fungi to the peptide. We found that tachyplesin I caused a rapid K^+ efflux from *Escherichia coli* cells, concomitant with a reduced cell viability. This result suggests that the peptide-induced permeability enhancement of the bacterial membranes may be a plausible action mechanism. Thus, we studied the interactions of tachyplesin I with various large unilamellar vesicles (LUVs) to reveal the molecular machinery of the antimicrobial activity. Tachyplesin I induced the leakage of calcein, a trapped fluorescent marker, from LUVs of acidic phospholipids, especially phosphatidylglycerol (PG), but not from phosphatidylcholine LUVs. A detailed analysis found that the affinity of the peptide to the PG membranes is very strong and that the binding of one peptide molecule to approx. 200 lipid molecules leads to a significant leakage. The location of tachyplesin I in membranes was estimated by use of the Trp-2 fluorescence of the peptide. The presence of PG LUVs caused a blue shift of the maximum wavelength, an increase in the quantum yield, and a complete protection from fluorescence quenching by an aqueous quencher, acrylamide. Moreover, the degree of fluorescence quenching of the Trp residue by *n*-doxylstearates was in the order $n = 5 > 7 > 12 \cong 16$. These results show that the Trp residue of tachyplesin I seems to locate in a hydrophobic environment near the surface of the PG bilayers.

Introduction

Tachyplesin I isolated from the acid extracts of hemocytes of a horseshoe crab (*Tachypleus tridentatus*), is a cyclic broad-spectrum antimicrobial peptide consisting of 17 amino acid residues [1–3]:

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Lys-Trp-Cys-Phe-Arg⁵-Val-Cys-Try-Arg
          |                  |     |
NH₂-Arg-Cys-Arg¹⁵-Arg-Try-Cys-Ile-Gly¹⁰
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This peptide has three tandem repeats of a tetrapeptide, hydrophobic amino acid-Cys-hydrophobic amino acid-Arg, suggesting an amphiphilic nature of the peptide. A two-dimensional NMR study [4,5] revealed that tachyplesin I forms a rigid antiparallel β-sheet structure, because of two intramolecular S-S linkages. The peptide binds strongly to bacterial lipopolysaccharide (LPS) and significantly inhibits the LPS mediated activation of factor C, the initiation factor in the limulus clotting cascade [2]. The binding to LPS cannot explain the susceptibilities of Gram-positive bacteria and fungi to the peptide, although the LPS binding ability will play some role in the antimicrobial activity against Gram-negative bacteria. Generally, amphiphilic peptides interact electrostatically and/or hydrophobically with phospholipid bilayers [6–14]. Especially, in the case of cyclic antimicrobial peptides resemblant to tachyplesin I, i.e., gramicidin S [8–10] and polymyxins [11,12], the interactions of the peptides with membranes seem to be important for their antimicrobial activities because a reduction in amphiphilic nature reduces the antimicrobial activity [8,11].

Abbreviations: LPS, lipopolysaccharide; LUV, large unilamellar vesicle; PC, egg yolk L-α-phosphatidylcholine; PS, bovine brain L-α-phosphatidyl-L-serine; PG, L-α-phosphatidylglycerol enzymatically converted from PC; DS, doxylstearate.

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Thus, in this paper, we aim to reveal the detailed antimicrobial machinery for tachyplesin I from the viewpoint of interactions with lipid membranes by use of various large unilamellar vesicles (LUVs). LUVs, a simple model for bacterial membranes, are a useful system for molecular level elucidation of peptide–lipid interactions. We can evaluate the binding ability of the peptide to the lipid membrane and the 'membrane-perturbing activity' of the peptide by monitoring the peptide induced leakage of an entrapped marker, calcein, from LUVs [5,6,15]. The location of the peptide in lipid bilayers can be estimated on the basis of the fluorescence of the unique Trp residue of the peptide [13,16–20]. We will discuss tachyplesin I–membrane interactions based on the results from the model membrane study with data on the peptide induced enhancement of *E. coli* membrane permeability.

Materials and Methods

Materials. Tachyplesin I was synthesized by Fmoc-based solid phase synthesis as described elsewhere [1]. The synthesized peptide after purification by HPLC was further refined by gel filtration (Sephadex G-15, 2.5 × 35 cm column, 4M acetic acid as an eluent), followed by lyophilization. The purity (approx. 100%) was determined by quantitative amino acid analysis.

Egg yolk L-α-phosphatidylcholine (PC) and bovine brain L-α-phosphatidyl-L-serine (PS) were purchased from Sigma. L-α-Phosphatidylglycerol (PG) enzymatically converted from PC was a gift from Nippon Fine Chemical Co. (Takasago, Japan).

5-, 7-, and 16-Doxylstearates (5-, 7-, and 16-DSs) were obtained from Sigma. 12-Doxylstearate (12-DS) was a product of Aldrich. These *n*-DSs were used as fluorescence quenchers. Calcein and spectrograde organic solvents were provided by Dojindo Laboratory (Kumamoto, Japan). All other chemicals from Wako were of special grade. Water was distilled twice from a glass still.

K^+ efflux from E. coli cells and cell viability. E. coli cells (ACTT 8739) were grown in a minimum essential medium (g/l) 10 polypepton/ 5 yeast extract/ 10 NaCl) at 37°C. The cells were harvested in the stationary phase of growth, washed twice with a Hepes buffer (10 mM Hepes/ 150 mM NaCl/ 0.1 mM KCl (pH 7.0)) and then resuspended in the Hepes buffer at $5 \cdot 10^8$ cfu/ml. The K^+ efflux from the cells after addition of tachyplesin I (final 10.2 μM) was monitored by a K^+-selective electrode connected to a microprocessor ion analyzer model 901 (Orion Research Inc.).

The cell viability was measured by a plate culture method on a petri dish with an agar medium (g/l) 15 agar/ 10 polypepton/ 5 yeast extract/ 10 NaCl). Samples (10 μl) collected at intervals diluted 100-fold immediately to stop the antimicrobial activity of the peptide and dispersed a 100 μl of the sample onto three petri dishes. The cell viability was determined by counting colonies after incubation for 20 h at 37°C.

Leakage from LUVs. The details of LUV preparation have been described elsewhere [21,22]. Briefly, aliquots of a lipid solution in chloroform/methanol (2:1, v/v) were placed in a round-bottom 20 ml flask. After the evaporation of the solvent by a rotating evaporator, the residual film was dried under vacuum overnight. The lipid film was hydrated with a 70 mM calcein solution (pH 7.0) or a buffer solution (10 mM Tris-HCl/ 150 mM NaCl/ 1 mM EDTA (pH 7.0)). The suspension was vortexed, and then extruded through polycarbonate filters successively (0.6 μm pore size × 6 times, 0.1 μm pore size × 10 times) by an extruder (Lipex Biomembranes Inc.). Untrapped calcein was removed by gel filtration (Sephadex G-50 medium, 1.5 × 30 cm column, the buffer being used as an eluent). The separated LUV fraction was appropriately diluted with the buffer and then mixed with calcein-free LUVs for obtaining the desired lipid concentration. The. lipid concentration was determined by phosphorus analysis [23].

The LUV suspension was mixed with a tachyplesin I/ buffer solution in a cuvette. The leakage of calcein out of the LUVs was monitored by measuring fluorescence intensity at 520 nm (excited at 490 nm) on a Shimadzu RF-5000 spectrofluorometer [24]. The fluorescence intensity corresponding to 100% leakage was determined by adding a 10 v/v% Triton X-100 solution (20 μl) to 2 ml of the sample.

Characterization of LUVs. An LUV suspension was diluted appropriately with the Tris buffer, followed by filtration (a cellulose nitrate filter of 8 μm pore size) to remove dust. The size distribution of the sample was measured on a Photal laser particle analyzer LPA-3100 connected to a photon correlater LPA-3000. All LUVs of different lipids had unimodal size distributions whose mean diameters were approximately 100 nm. The vesicles prepared in this manner had a trapped volume of 1.3 l/mol phospholipid (calcein being employed as an aqueous marker), coinciding with a reported value for an 100-nm sized LUV [21]. Moreover, a fluorescence quenching experiment by using dansyl-phosphatidylethanolamine and $CuSO_4$ supported the unilamellarity [22].

Fluorescence. Fluorescence spectra of the Trp residue of tachyplesin I (1 μM in the Tris buffer, excited at 280 nm) were measured on titration of the peptide solution with small aliquots of a PG or PC LUV suspension (5 mM) in the cuvette. Fluorescence spectra were corrected according to Melhuish's method [25] with a modification after subtraction of the corresponding blank spectra.

Fluorescence quenching was studied as follows: an LUV solution was mixed with a tachyplesin I (final 1

μM)/ buffer solution in a cuvette (2 ml). Maximum Trp fluorescence intensities (at 352 nm in the absence of the LUVs, at 336 nm in the presence of the LUVs, excited at 280 nm) were recorded on titration of the solution with small aliquots of fluorescence quencher solutions (2 mM *n*-DSs in methanol or 5 M acrylamide in water) in the cuvette.

All the experiments were carried out at 30 ± 0.5°C.

Results

K^+ efflux from E. coli cells

Fig. 1 shows that addition of tachyplesin I to *E. coli* cells induced a rapid K^+ efflux from the cells, concomitant with a reduced cell viability. Almost all cells were died within five minutes. We, however, observed neither any K^+ efflux nor the cell viability reduction over 30 min prior to addition of the peptide (data not shown). These results suggest that the peptide induced enhancement of bacterial membrane permeability may cause the antimicrobial action as in the case of gramicidin S [26,27], polymyxin B [11], and sarcotoxin I [28].

Calcein leakage from LUVs.

Fig. 2 illustrates the effects of lipid species on the leakage of calcein entrapped within LUVs. Addition of 5.1 μM tachyplesin I to a PS LUV suspension led to a leakage. A more significant leakage was observed in the case of a PG LUV suspension at a peptide concentration as low as 0.85 μM. In contrast, a PC LUV suspension leaked little calcein on addition of the peptide at a higher concentration (8.5 μM). These results show that tachyplesin I interacts with only negatively charged phospholipids.

We further investigated quantitatively the tachyplesin I induced leakage from PG LUVs to evaluate the affinity of the peptide to the LUVs and the amount of the membrane-bound peptide necessary for the leakage. These factors can be estimated by analyzing

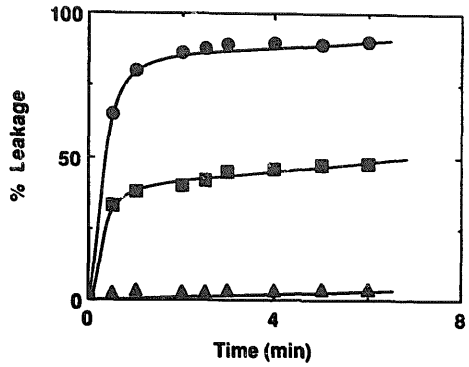

Fig. 2. Effects of lipid species on tachyplesin I induced calcein leakage out of LUVs at 30 °C. Percent leakage is plotted as a function of time. The peptide concentration: 0.85 μM for PG (●); 5.1 μM for PS (■); 8.5 μM for PC (▲). The lipid concentrations are 42–48 μM.

both the peptide concentration and the lipid concentration dependencies of the leakage rate [6,7,15]. Fig. 3 shows dose-response curves for the leakage rate, expressed as percent leakage for the initial 1 min after the peptide addition, at different lipid concentrations. A rise in the lipid concentration shifts the curve to the right, suggesting that the binding of tachyplesin I to the LUVs is involved in the leakage process. We assumed that the leakage rate is determined only by the amount of the membrane-bound peptide per lipid molecule, r. The value of r can be connected to the total peptide concentration, $[P]_0$ and the lipid concentration, [L] through a material balance equation.

$$[P]_0 = [P]_f + r[L]$$

where $[P]_f$ stands for the free peptide concentration. The r value and the corresponding $[P]_f$ value can be estimated with at least three sets of $[P]_0$ and [L], where a given leakage rate was observed in Fig. 3. A $[P]_0$ vs. [L] plot at any given leakage rates gave linear relations from which we can estimate $[P]_f$ and r (Fig. 4, the correlation factors were greater than 0.97). The $[P]_f$

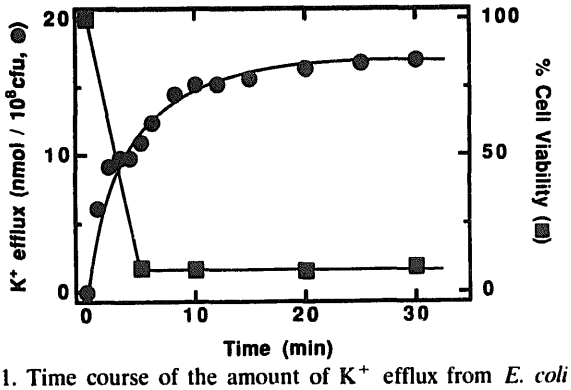

Fig. 1. Time course of the amount of K^+ efflux from *E. coli* cells and the cell viability after treatment of tachyplesin 1 Tachyplesin I (10.2 μM) was added to an *E. coli* (ATCC 8739) cell suspension ($5 \cdot 10^8$ cfu/ ml) at the zero time. The K^+ efflux (●) and the percent cell viability (■) were monitored at intervals.

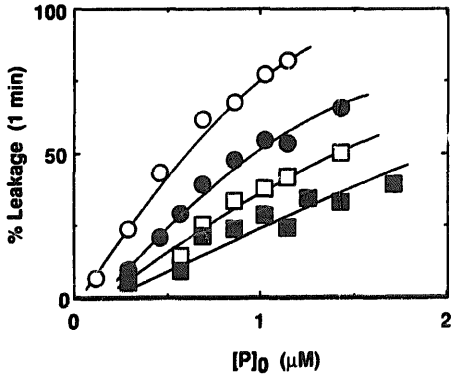

Fig. 3. Dose-response curves for tachyplesin I induced calcein leakage out of LUVs at 30°C. Percent leakage for the initial 1 min is shown as a function of applied peptide concentration $[P]_0$. The lipid concentration: ○, 59.5 μM; ●, 110 μM; □, 157 μM; ■, 209 μM.

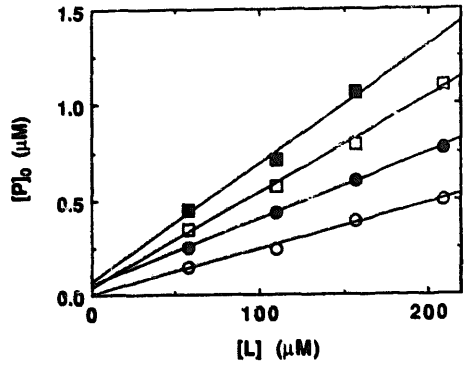

Fig. 4. Determination of free and membrane-bound tachyplesin I concentrations. At least three pairs of $[P]_0$ and [L] values where a given leakage rate was observed were obtained from Fig. 3. $[P]_0$ is plotted against [L] according to the equation in the text. Leakage rate (the percent leakage for the initial 1 min): ○, 10%; ●, 20%; □, 30%; ■, 40%.

values were almost zero (less than 60 nM) at any leakage rates. This result demonstrates that the peptide binds very strongly to the PG membrane. Fig. 5 shows the relationship between the leakage rate and r, suggesting that the calcein leakage was observed at r values larger than 0.002. We acquired very similar results when we used percent leakage for the initial 5 min instead of the initial 1-min data, guaranteeing the validity of our analysis (data not shown).

Trp residue fluorescence

Tachyplesin I has the unique Trp residue whose fluorescence can be exploited for estimating the location of the chromophore in bilayers, because the fluorescence spectrum of Trp residue is known to be sensitive to environments around the residue [29–33]. Addition of PG LUVs (10 μM) to the peptide (1 μM) caused a blue shift of maximum fluorescence wavelength from 352 nm to 336 nm, concomitant with an increase in quantum yield (Fig. 6b). Further addition of PG LUVs up to 100 μM did not change the spectrum. In contrast, addition of PC LUVs up to 100 μM changed neither the maximum fluorescence wavelength

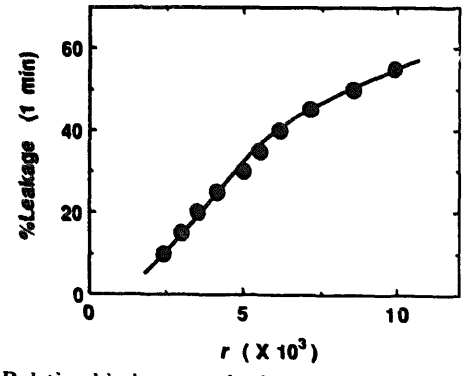

Fig. 5. Relationship between the leakage rate (percent leakage for the initial 1 min) and the amount of membrane-bound tachyplesin I per lipid, r.

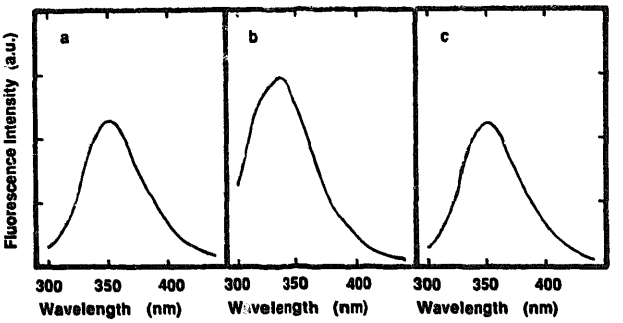

Fig. 6. Change in Trp fluorescence spectra upon binding to membrane at 30 °C. Corrected fluorescence spectra (excitation at 280 nm) of tachyplesin I (1 μM) were recorded (a) in the Tris buffer and in the presence of (b) PG LUVs (10 μM) and (c) PC LUVs (100 μM).

nor the quantum yield. These results suggest that the peptide binds very strongly to the PG LUVs but never to the PC LUVs, coinciding the leakage data (Fig. 2). Moreover, the Trp residue of tachyplesin I seems to be buried in a hydrophobic environment of the PG bilayers. Thus, we next carried out fluorescence quenching experiments to estimate the location of the Trp residue in the membrane.

Fluorescence quenching

Fig. 7 shows the Stern-Volmer plots [34] of the Trp fluorescence quenching by acrylamide in the presence or absence of PG LUVs. Inner filter effects due to an absorption of the excitation beam by acrylamide ($\varepsilon^{280} = 4.3\ M^{-1}\ cm^{-1}$) have been corrected [18]. In the buffer, the fluorescence of the Trp residue was quenched according to the Stern-Volmer equation [34] with a Stern-Volmer constant of 8.7 M^{-1}, the value being somewhat smaller than values (12–17 M^{-1}) reported for short peptides [18]. The presence of an adjacent disulfide linkage, which would shorten the life time of the fluorophore and sterically reduce the effective local quencher concentration around it, may cause

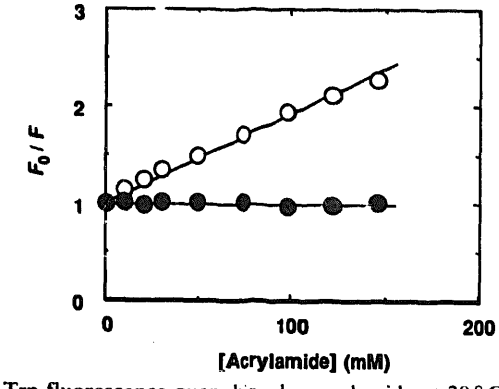

Fig. 7. Trp fluorescence quenching by acrylamide at 30 °C. A tachyplesin I (1 μM) solution was titrated with acrylamide in the absence (○) or the presence (●) of PG LUVs (100 μM). The ratio of the fluorescence maximal intensity (excitation at 280 nm) in the absence of the quencher, F_0 to the intensity in the presence of the quencher, F is plotted as a function of the quencher concentration.

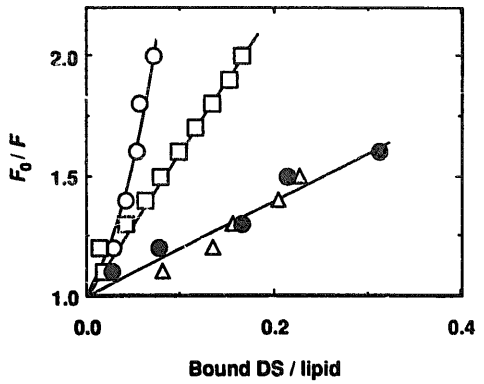

Fig. 8. Trp fluorescence quenching by *n*-DSs at 30 °C. The ratio of the fluorescence maximal intensity (excitation at 280 nm) in the absence of the quencher, F_0 to the intensity in the presence of the quencher, F is plotted as a function of the amount of the membrane-bound quencher per lipid [16]. The quencher: ○, 5-DS; □, 7-DS; △, 12-DS; ●, 16-DS.

the weaker dynamic quenching and the absent static quenching [35]. The presence of the PG LUVs completely protected the Trp residue from the quenching, indicating the fluorophore is located in an aqueous quencher-inaccessible hydrophobic environment of the bilayers. We further investigated the location of the Trp residue with *n*-DSs.

The fluorescence quenching by *n*-DSs can be used to estimate the transversal location of a Trp residue in membranes [16,17,19]. Reportedly, the series of *n*-DSs differs in the affinity to lipid membranes [16]. Thus, the quenching efficiency of *n*-DSs must be compared on the basis of the amount of the membrane-bound quencher per lipid molecule. This amount was obtained by analyzing dependencies of the quenching efficiencies on both the quencher and the lipid concentrations [16]. Four different lipid concentrations were used for each *n*-DS quenching experiment. Fig. 8 shows the amount of the bound *n*-DS per lipid molecule vs. F_0/F plots. The quenching efficiencies were in the order 5-DS > 7-DS > 12-DS ≅ 16-DS. This result suggests that the Trp residue locates near the surface of the PG bilayers.

Discussion

Tachyplesin I exhibits broad spectrum antimicrobial activities [2,3]. The strong binding of the peptide to LPS cannot explain activities for gram positive bacteria and fungi. Gram positive bacteria, however, have (lipo) teichoic acids and teichuronic acids which may have similar binding characteristics as LPS. An NMR study [4,5] revealed that tachyplesin I conforms to an amphiphilic antiparallel β-sheet structure, suggesting interactions with bacterial membranes as in the case of gramicidin S [9,26,27] or polymyxin B [11]. Our results (Fig. 1) are compatible with this assumption, although these data alone do not prove the above mechanism [11]. Thus, we examined in detail the interactions of tachyplesin I with lipid bilayer membranes, a model system for biomembranes.

Tachyplesin I specifically interacts with acidic phospholipid membranes as revealed by the permeability enhancement (Fig. 2) and the Trp fluorescence spectra (Fig. 6). The peptide seems not to bind to neutral membranes probably because of a strong hydrophilicity of the peptide bearing a net positive charge as large as +7. In contrast, gramicidin S, which possesses two positive charges, can bind and disrupt phosphatidylcholine bilayers [10,27].

Our quantitative analysis elucidated that tachyplesin I binds very strongly to PG bilayers. (An apparent partition coefficient, $K_{app} = r/[P]_f$, is greater than 10^7 M^{-1}). An enhancement of the membrane permeability occurs at r values around 0.005. The membrane-perturbing activity is fairly high compared with that of a hypelcin A-egg yolk phosphatidylcholine system ($r \cong 0.01$) [15] or that of a magainins-acidic phospholipid system ($r \cong 0.02$–0.1) [7], although in the latter two systems small unilamellar vesicles were used. We are studying the effects of vesicle curvature on peptide-lipid interactions.

Fluorescence of an intrinsic tryptophan residue in peptides or proteins can be utilized for estimating the location of the fluorophore in membranes [13,16–20,35]. Our fluorescence data (Figs. 6 and 7) indicate that the unique Trp − 2 residue of tachyplesin I is located in an aqueous quencher inaccessible, hydrophobic environment of the PG bilayers. The complete inaccessibility is in agreement with the very strong binding of the peptide to the membranes (Fig. 4), that is, all peptide molecules present are membrane-bound. The doxylstearate quenching experiment (Fig. 8) demonstrated that 5-DS quenches the Trp fluorescence much more effectively than 12- or 16-DS does, in spite of more fluid environments around the latter two quenchers. We can conclude that the Trp residue is buried in a hydrophobic environment near the surface of the bilayers. This location is compatible with a plausible model in which the amphiphilic β-sheet peptide binds to the membrane surface with the molecular plane parallel to the surface. The orientation of the peptide in the membranes is a subject of further study.

The leakage mechanism is unknown at the present stage. It may be the peptide induced defect of lipid packing or the partial solubilization of lipids by the peptide. We will investigate lipid conformations using some spectroscopic and thermal techniques.

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