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Interaction of Bundled Ser-rich Amphiphilic Peptides with Phospholipid Membranes

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Abstract: To investigate properties of hydrophilic bundled peptides and their interactions with phospholipid membranes, bundled peptides named [Trp^{2}]- and [Trp^{12}]-4α-4_6S9, which are composed of four fragments of amphiphilic 24-mer peptide, were designed and synthesized. Tryptophan (Trp) was introduced at the 2nd position from the N-terminal or at the centre (12th) of the helix to monitor the peptide–lipid interaction. Circular dichroism measurements indicated that the peptides had low α-helicities in a buffer solution (pH 7.4) and also in the presence of dipalmitoyl-DL-3-phosphatidylcholine (DPPC) vesicles. In the presence of DPPC/dipalmitoyl-DL-3-phosphatidylglycerol (DPPG) (3:1) vesicles, the measurement could not be taken because of turbidity induced by vesicle aggregation. Both peptides had moderate perturbation activity for both the neutral and acidic vesicles at 25°C. The perturbation patterns at 50°C were much different from those at 25°C and the maximum activity reached 100% at a low peptide concentration. The results of the measurement of membrane fusion activity of peptides showed a similar tendency to that found in the perturbation experiment. A quenching experiment indicated that the Trp^{2} and Trp^{12} residues in [Trp^{2}]- and [Trp^{12}]-4α-4_6S9 were scarcely embedded in neutral lipid membranes. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: bundled peptide; amphiphilic structure; membrane perturbation; membrane fusion; Trp titration

INTRODUCTION

The many bioactivities of naturally occurring amphiphilic α-helical peptides include antibiotics, self-defence, hormonal function and ion channels. The relationship between their structures and functions is an interesting subject from the peptide chemistry and pharmacology viewpoint. Therefore, various kinds of amphiphilic α-helical peptides have been synthesized to clarify the structure–function relationships and to create functionally or structurally unique peptides, e.g. membrane-interacting peptides [1–3], conformationally constrained peptides [4–6] and bioactive peptides [7,8]. On the other hand, the design of bundled peptides have also become interesting in recent years, because such peptides are useful not only as models of natural polypeptides, but also as functional materials. Examples of bundled peptides include various kinds of stranded polypeptides [9–11] and bundled peptides containing non-peptidyl components [12–14].

We previously reported that a 24-mer peptide, H-(Leu-Ala-Arg-Leu)$_3$-(Leu-Arg-Ala-Leu)$_3$-OH (4_6), which was designed to have an amphiphilic α-helical structure, showed moderate membrane-perturbing activity toward phospholipid membranes

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Abbreviations: Boc, tert-butyloxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; CD, circular dichroism; CF, 5(6)-carboxyfluorescein; DCM, dichloromethane; DMF, dimethylformamide; DPPC, dipalmitoyl-DL-3-phosphatidylcholine; DPPG, dipalmitoyl-DL-3-phosphatidylglycerol; Fmoc, 9-fluorenylmethoxycarbonyl; Gu HCl, guanidine hydrochloride; HOBt, 1-hydroxybenzotriazole; MBHA, 4-methylbenzhydrylamine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazo-4-yl)dipalmitoyl phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)dipalmitoyl phosphatidylethanolamine; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

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and formed cation-selective ion channels on a planar membrane [15]. The bundled peptide 4α-4_6, consisting of four fragments of the 4_6 chain, showed a much stronger activity than 4_6. Furthermore, the bundled peptide induced vesicle fusion at a lower concentration than that of the leakage. Due to very strong perturbation activity, 4α-4_6 could not form stable ion channels, indicating that bundling of basic amphiphilic α-helical peptides greatly enhances the activity toward lipid membranes. Another peptide, 4α-4_6S, in which 24 Ser residues were present instead of the hydrophobic Leu and Ala residues found in 4α-4_6, also showed similar activities to those of 4α-4_6 in spite of an increase in the hydrophilic region from 1/4 in 4α-4_6 to 1/2 in 4α-4_6S [16].

In this study, we prepared more hydrophilic peptides [Trp^2]-4α-4_6S9 and [Trp^{12}]-4α-4_6S9 that contain 36 Ser residues (Figure 1) and examined the effects of these hydrophilic peptides on lipid membranes. Tryptophan (Trp) was introduced into the 2nd position from the N-terminal, or at the centre (12th position) to monitor the peptide–lipid interaction. The secondary structures of the peptides were determined by circular dichroism (CD) measurement, and their effects on lipid membranes were evaluated by membrane-perturbation and -fusion measurements. A fluorescence study of the peptides was also carried out.

MATERIALS AND METHODS

Materials

Reagents used for the synthesis and analysis were of reagent grade. Peptides were synthesized manually using the Boc strategy in a glass vessel. Amino acid derivatives and the resin were purchased from Watanabe Chemical (Hiroshima, Japan). Carboxyfluorescein from Kodak (Rochester, NY) was purified twice by recrystallization from ethanol. DPPC and DPPG were purchased from Sigma (St. Louis, MO), NBD-PE and Rh-PE were from Avanti Polar Lipids (Alabaster, AL). Fluorescence data were measured using a Hitachi spectrofluorimeter F-3010 with a thermostatted cell holder. Amino acids were analysed by the ninhydrin method using a JEOL JLC-300 analyzer after hydrolysis of the peptides at 110°C for 24 h in 6 M HCl.

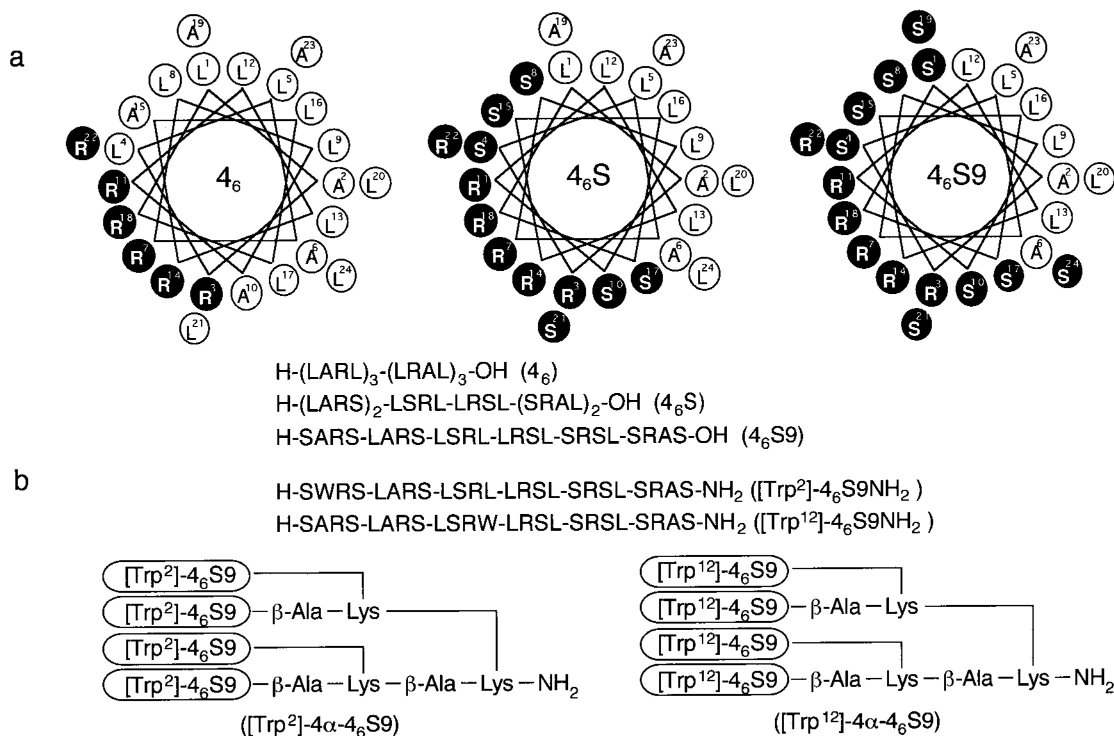

Figure 1 Structures of the amphiphilic α-helical peptides. (a) Helical wheels and primary sequences of model peptides, 4_6, 4_6S and 4_6S9. Open and filled circles show hydrophobic and hydrophilic residues, respectively. (b) Primary sequences of newly designed peptides, [Trp^2]- and [Trp^{12}]-4_6S9NH_2, and illustration of bundled peptides, [Trp^2]- and [Trp^{12}]-4α-4_6S9.

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Synthesis of Peptides

[Trp^2]-4α-4_6S9·28AcOH: Fmoc-Lys(Boc)-OH (0.2 mmol) was introduced into the MBHA resin (0.5 g; 0.79 mmol/g resin) [17] by the BOP-HOBt method [18,19] to give a Fmoc-Lys(Boc)-resin with a rather low substituted level (0.1 mmol/g). After the Fmoc group was removed with 20% piperidine/DMF for 15 min, Boc-βAla-OH (0.3 mmol) was coupled using the same method. The Boc group was removed with 50% TFA/DCM for 20 min. Into the resulting H-βAla-Lys-resin were introduced Fmoc-Lys(Boc)-OH and Boc-βAla-OH in that order, to produce the Boc-dendrimer peptidyl resin, Boc-βAla-Lys(Boc)-βAla-Lys[Boc-βAla-Lys(Boc)]-resin. The bundled peptide was synthesized starting from this peptidyl resin (0.5 g, 0.05 mmol) as described by Sakamoto *et al.* [16]. The Boc-amino acid derivatives used were Ala, Arg(Tos), βAla, Leu, Lys(Boc), Ser(Bzl) and Trp(CHO). The crude peptide was purified twice by passing it through a column of Sephadex G-50 (2.5 × 90 cm) with 10% AcOH and produced a peak on the chromatography. After a further two purifications by reversed-phase high performance liquid chromatography (RP-HPLC) with Wakosil 5C4 (4.6 × 100 mm) (A: 100% H_2O/0.1% TFA, B: 100% CH_3CN/0.08% TFA, linear gradient from 0 to 100% of B for 30 min, 3 ml/min), fractions in retention time of 18–20 min were collected at a yield of 41 mg (4.4%). The ammo acids analysed were Ser (36) 29.8, Ala (8) 8.0, Leu (24) 22.4, Lys (3) 3.3 and Arg (24) 21.9. The purified peptide was identified by MALDI-TOF MS; calculated 11528 Da; found 11533 Da. [Trp^{12}]-4α-4_6S9·28AcOH: this compound was synthesized as described by Sakamoto *et al.* [16]. The yield was 60 mg (5.3%). The amino acids analysed were Ser (36) 29.8, Ala (12) 12.0, Leu (20) 22.2, Lys (3) 3.8 and Arg (24) 21.4. MALDI-TOF MS; calculated 11359 Da; found 11351 Da.

Preparation of Phospholipid Vesicles

Small unilamellar vesicles of DPPC and DPPC/DPPG (3:1) were prepared for CD, fluorescence, leakage and fusion experiments [16]. Phospholipid (7.5 mg) was dissolved in $CHCl_3$ and MeOH (2 ml) and was then dried by a stream of nitrogen gas. The dried lipid was hydrated in 20 mM Tris–HCl buffer (pH 7.4, 10 ml) using a Branson bath-type sonicator. The suspension was sonicated at 50°C for 20 min using a Titec Ultrasonic Processor VP-5T at 10 W intensity. The vesicles were left for 30 min at 25°C before the measurements. The lipid concentration was 1.0 mM. The vesicles trapping CF were prepared using the procedure described by Sakamoto *et al.* [16]. The dried lipid (20 mg) was hydrated in 20 mM Tris–HCl buffer (pH 7.4, 2.0 ml) containing 0.10 M CF. The CF-entrapped vesicles were separated from the free CF by gel filtration using Sephadex G-75 (1.0 × 20 cm) with the same buffer (pH 7.4).

CD Measurement

CD spectra were recorded on a JASCO J-720W spectropolarimeter with a thermostatted cell holder using a quartz cell of 1.0 mm path length. The peptide concentration was 5.0 μM in solution or in the presence of phospholipid vesicles (0.90 mM). Measurements were made at 25 or 50°C. The percentage helicity of the peptides was calculated with the following equation: % helicity $= 100 \times [\theta]_{222}/(-40000 \times (1-2.5/n) + 100 \times t)$, where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm, and n and t are number of residues and temperature (°C), respectively [20].

CF Leakage

The dye-leakage experiment was carried out using the procedure described by Lee *et al.* [21]. In a cuvette, 20 μl of the vesicles containing 0.1 M CF was added to 20 mM Tris–HCl buffer (pH 7.4, 2.0 ml) to give a vesicle solution with a final lipid concentration of 70 μM. To a cuvette placed in a holder (25 or 50°C) was added 20 μl of an appropriate dilution of the peptides in the buffer. The fluorescence intensities of CF were monitored at 510 nm (excited at 470 nm) and were measured 2 min after the addition of the peptides. To measure the fluorescence intensity for 100% dye release, 10 μl of Triton® X-100 (10% in Tris buffer) was added to dissolve the vesicles. The percentage of the dye release caused by the peptides was evaluated by the equation: $100 \times (F - F_0)/(F_t - F_0)$, where F is the fluorescence intensity achieved by the peptides, and F_0 and F_t are intensities observed without the peptides and after Triton® X-100 treatment, respectively.

Fusion Assay

Two kinds of vesicles, one containing NBD-PE and the other Rh-PE (2.0 mol% each), were prepared by sonication as described by Lee *et al.* [21]. Equimolar amounts of the vesicles were mixed at 25°C and the final lipid concentration was adjusted to 70 μM. The peptides were added to the mixed vesicles and the decrease in NBD fluorescence was recorded

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continuously at an excitation wavelength of 450 nm and an emission wavelength of 530 nm. The fluorescence intensity (F) was measured 5 min after adding the peptides. The fluorescence intensity (F_t) for 100% fusion was obtained using the vesicles in which NBD-PE and Rh-PE were pre-mixed. The fusion percentage was evaluated by the equation: $100 \times (F_0 - F)/(F_0 - F_t)$, where F is the fluorescence intensity achieved by the peptides, F_0 is the intensity observed without the peptides and F_t is the intensity observed in the presence of NBD-PE and Rh-PE.

Trp Titration

To a solution of 5 μM peptide in the buffer (1.0 ml), was added 1 mM DPPC vesicles (5–20 μl) in the buffer at 25°C. After 5 min, the fluorescence intensity of Trp was scanned from 300 to 400 nm (excited at 280 nm).

RESULTS AND DISCUSSION

Peptide Design and Synthesis

As shown in Figure 1(a), 4_6 contains six Arg residues and 4_6S has six Arg and six Ser residues as hydrophilic components. These peptides could form amphiphilic α-helical structures. The bundled peptide, in which four segments of 4_6 are bundled on a template, had strong membrane-perturbing activity. Furthermore, the bundled peptide of 4_6S also showed a similar property, although it was more hydrophilic than the 4_6 derivative [16]. We therefore designed more hydrophilic peptides to examine their properties and their activity toward lipid vesicles. The structures of linear and bundled peptides newly synthesized in this study are shown in Figure 1(b). Hydrophilic amino acid residues comprise 2/3 of the total residues. Among various types of templates [22–26], a dendrimer-type core composed of Lys residues, which was originally introduced by Tam [25] was selected for easy preparation and was modified with a flexible anchor of βAla residues. To monitor the peptide–lipid interaction, a Trp residue was introduced at the 2nd position from the N-terminal or at the centre (12th) of the peptide. Synthesis was carried out using the solid phase method on MBHA resin. The substitution level of Fmoc-Lys(-Boc)-OH was reduced (0.1 mmol/g resin) to increase the coupling efficiency in the step of bundle peptides. The synthesized peptides were purified by gel chromatography on Sephadex G-50 (10% AcOH) followed by RP-HPLC with Wakosil 5C4 (A, 100% H_2O/0.1% TFA; B, 100% CH_3CN/0.08% TFA). |Gel chromatography showed a similar retention volume of the purified peptides (Mw = 11 500 for [Trp[2]]-4α-4_6S9 and 11 400 for [Trp[12]]-4α-4_6S9) to that of the authentic sample 4α-4_6 (Mw = 11 300) and a sharp peak on RP-HPLC was also observed. The purified peptides were identified by MALDI-TOF MS.

CD Study

The CD was measured in 20 mM Tris–HCl buffer (pH 7.4), TFE and in the presence of DPPC vesicles. For the comparison, several previously prepared linear (4_6, 4_6S) and bundled peptides (4$\alpha$$4_6$, [Trp[12]]-4$\alpha$-$4_6$S) were also used [16]. In the presence of DPPC/DPPG (3:1) vesicles, correct measurements could not be taken for all the peptides because of turbidity. Figure 2 shows the CD patterns of the 4_6S9-series peptides and Table 1 lists the helical contents of all the peptides. In the buffer solution (pH 7.4), the linear peptides [Trp[2]]- and [Trp[12]]-4_6S9 NH_2 were mainly random. The bundled peptides [Trp[2]]- and [Trp[12]]-4α-4_6S9 showed α-helical curves, but the helical contents were low (Figure 2(a)). The α-helical content decreased with the increasing content of Ser residue in peptides (Table 1). The helical content of 4α-4_6 did not vary with a change in temperature, while that of the Ser-containing peptides decreased with a rise in temperature in the buffer [16]. These results suggest that because of the low α-helical propensity of Ser [27], the structures of the Ser-containing peptides are not as stable as those of 4_6 and 4α-4_6 in an aqueous environment. In TFE, all the peptides except for 4α-4_6 showed similar α-helical contents (Figure 2(b)), indicating that these peptides may have similar conformations. The CD curves of peptides in the presence of DPPC were similar to those in aqueous solution at 25°C (Figure 2(c)). The peptide conformation might be safely kept in the presence of DPPC at high temperature because the helical contents were not much changed at 50°C. It is probable that the hydrophobic interaction between peptide and neutral lipid assists the holding of the peptide conformation.

To compare stability of the 4_6S9-series peptides with that of 4α-4_6, the CD of [Trp[12]]-4α-4_6S9 and 4α-4_6 were measured in the presence of guanidine hydrochloride (GuHCl) and the mean residue ellipticity at 222 nm, $[\theta]_{222}$, was plotted as a function of GuHCl concentration (Figure 3). An increase in GuHCl concentration caused a gradual decrease in $[\theta]_{222}$ of [Trp[12]]-4α-4_6S9, which completely disappeared at a concentration of 6 M. On the other hand, change in the $[\theta]_{222}$ for [Trp[12]]-4α-4_6 was negligible. These results also indicate that the stability of the

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Table 1 α-Helical Contents of Linear and Bundled Peptides

Peptide	α -Helical content (%)				
	Buffer (pH 7.4)		TFE	DPPC	
	25°C	50°C	25°C	25°C	50°C
Linear peptide					
4_6	75	62	62	76	80
4_6S	23	15	67	40	32
[Trp^2]-4_6S9	16	8	65	21	12
[Trp^{12}]-4_6S9	16	7	69	20	14
Bundled peptide					
4α-4_6	83	82	85	89	95
[Trp^{12}]-4α-4_6S	38	22	64	51	45
[Trp^2]-4α-4_6S9	27	19	62	29	32
[Trp^{12}]-4α-4_6S9	27	20	55	32	31

Ser-containing peptides is clearly weaker in water compared with that of 4α-4_6.

CF Leakage

The CF leakage experiments were done using DPPC and DPPC/DPPG (3:1) vesicles at 25 and 50°C (Figure 4). In the presence of neutral DPPC vesicles at 25°C, some characteristics of the peptides in the CF leakage activity were observed (Figure 4(a)). First, the activities of linear peptides and bundled peptides were distinctly different. In our previous study, we observed the same tendency using 4α-4_6 and 4_6, which were used as standard peptides in this study, i.e. bundling of linear amphiphilic peptides with leakage activity remarkably enhances the activity [16]. Second, hydrophobicity contributed to the CF leakage. Peptide 4α-4_6 generally showed stronger activity than the Ser-containing peptides, this means that hydrophobic interaction between peptide and neutral lipid is important for membrane perturbation. A slightly different leakage pattern was found in the presence of acidic DPPC/DPPG (3:1) vesicles (Figure 4(b)). At 25°C, the leakage activities of 4α-4_6 and [Trp^{12}]-4α-4_6S were fairly weaker than those in the presence of DPPC at low peptide concentrations, while the activities of [Trp^2]- and [Trp^{12}]-4α-4_6S9 were unchanged.

Above the phase transition temperature of lipids (50°C), the leakage patterns differed much more from those at 25°C. With DPPC, the CF leakage

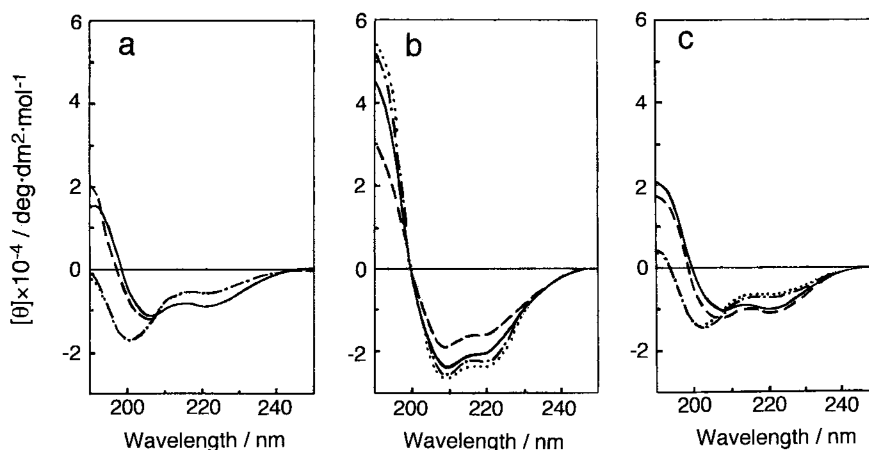

Figure 2 CD spectra of [Trp^2]-4α-4_6S9 (—), [Trp^{12}]-4α-4_6S9 (– – –), [Trp^2]-4_6S9NH$_2$ (-·-·-), and [Trp^{12}]-4_6S9NH$_2$ (·····) in 20 mM Tris–HCl buffer (pH 7.4) (a), TFE (b), and in the presence of DPPC(c). [Peptide] = 5 μM, [Lipid] = 1 mM.

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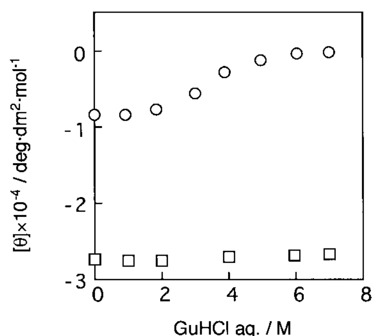

Figure 3 Stability of peptides in aqueous GuHCl at 25°C. (□) 4α-4_6, (○) [Trp[12]]-4α-4_6S9. [Peptide] = 5 μM.

reached the maximum at the peptide concentration of *ca.* 0.06 μM (Figure 4(c)). The order of the activity was 4α-4_6 > [Trp[12]]-4α-4_6S > [Trp[2]]-, [Trp[12]]-4α-4_6S9 and a clear difference in the activities between the bundled peptides and the linear peptides was still observed. We believe that the hydrophobic region of the bundled peptides easily interacts with the less ordered methylene chains of lipids to penetrate into the membranes and disturb them. A similar result was obtained in the presence of DPPC/DPPG (3:1) vesicles at 50°C (Figure 4(d)). However, it is noticeable that the peptide concentration that induces the maximum activity was reduced to 0.2–0.3 μM compared with that of *ca.* 0.06 μM in the presence of DPPC vesicles. As described by Dathe *et al.* [2], the electrostatic interaction between the basic peptide and the acidic head group of the lipid may have inhibited further penetration by the peptide into the hydrophobic region of the membrane, resulting in weaker destabilization of the membrane.

Antibacterial Activity

Antibacterial activity of the peptides was examined for reference by the standard agar dilution method as described by Mihara *et al.* [28]. None of the bundled peptides showed growth inhibitory activity at peptide concentrations of less than 100 μg/ml against Gram-positive and -negative bacteria, although they had a strong membrane-perturbing activity for DPPC and DPPC/DPPG (3:1) vesicles at 50°C. The membrane-perturbing activity of a short model peptide, Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$, was weaker compared with that of the bundled peptides. Nevertheless, the short peptide showed a strong antibacterial activity against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* [28]. It can be said that activities observed for a lipid membrane do not always reflect those of the cell membrane.

Membrane Fusion Activity

Fusion of phospholipid bilayers by peptides was determined by intermixing phospholipids between vesicles containing either NBD-PE or Rh-PE [21]. The intermixing of the chromophoric phospholipids caused quenching NBD fluorescence at 530 nm by the energy transfer to rhodamine (Figure 5). [Trp[2]]- and [Trp[12]]-4α-4_6S9 caused the maximum fusion of the DPPC vesicles at the peptide concentration of 0.1 μM (Figure 5(a)), which was slightly lower than that required for the CF leakage activity at 25°C. No noticeable difference in the activities between the 4α-46_S9 and 4α-4_6S peptides was observed. The degree of maximum fusion was about 50% for [Trp[2]]- and [Trp[12]]-4α-4_6S9 and [Trp[12]]-4α-4_6S, while 4α-4_6 showed a maximum fusion of 60% at 0.03 μM. In the presence of DPPC/DPPG (3:1) vesicles at 25°C, the peptide concentrations required for the maximum fusion were four to five times those in the presence of DPPC vesicles. However, almost complete fusion was observed at 0.4 μM, except in the case of 4α-4_6, which showed complete fusion at 0.18 μM (Figure 5(b)). The results of the leakage and fusion measurements indicate that membrane perturbation and fusion occur competitively in the presence of DPPC at 25°C, whereas fusion precedes leakage in the presence of DPPC/DPPG (3:1). The basic peptide that binds to the surface of acidic vesicles may help to attract vesicles nearby rather than perturb the lipid membrane.

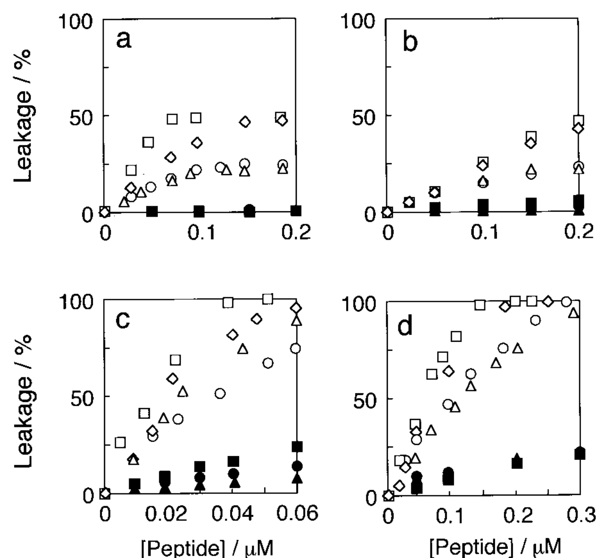

Figure 4 Profiles of carboxyfluorescein leakage. (a) DPPC at 25°C, (b) DPPC/DPPG (3:1) at 25°C, (c) DPPC at 50°C, (d) DPPC/DPPG (3:1) at 50°C. (□) 4α-4_6, (△) [Trp[2]]-4α-4_6S9, (○) [Trp[12]]-4α-4_6S9, (■) 4_6, (▲) [Trp[2]]-4_6S9NH$_2$, (●) [Trp[12]]-4_6S9NH$_2$, (◇) [Trp[12]]-4α-4_6S. [Lipid] = 70 μM.

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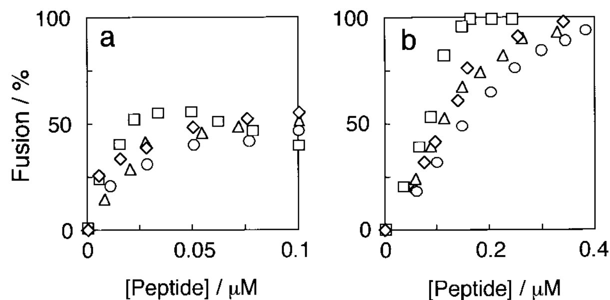

Figure 5 Profiles of the vesicle fusion. (a) DPPC at 25°C, (b) DPPC/DPPG (3:1) at 25°C. (□) 4α-4_6, (△) [Trp^2]-4α-4_6S9, (○) [Trp^{12}]-4α-4_6S9, (◇) [Trp^{12}]-4α-4_6S. [Lipid] = 70 μM.

Trp Titration

The fluorescence properties of the Trp residues incorporated into the 4α-4_6S9 peptides were also examined in the presence and absence of DPPC vesicles (Figure 6), whereas correct measurements could not be taken in the presence of DPPC/DPPG (3:1) vesicles because of slight turbidity. In the aqueous solution, the maximum wavelengths of [Trp^2]- and [Trp^{12}]-4α-4_6S9 were 344 and 345 nm, respectively. The maximum wavelengths were little affected by adding lipid, indicating that both Trp residues might be exposed to the aqueous phase and that the bundled peptides do not perpendicularly insert into the lipid bilayers. We previously reported that [Trp^1]- and [Trp^{12}]-4α-4_6S were rather perpendicularly embedded in the membranes because the maximum fluorescence changed to a longer wavelength in [Trp^1]-4α-4_6S with increasing lipid concentration, whereas the maximum fluorescence changed to a shorter one in [Trp^{12}]-4α-4_6S [16].

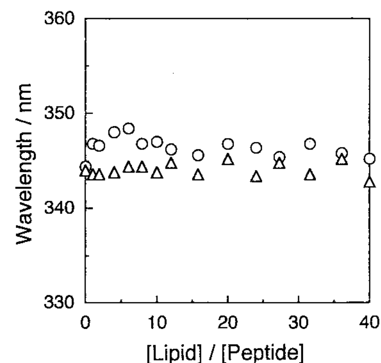

Figure 6 Fluorescence wavelength of [Trp^2]-4α-4_6S9 and [Trp^{12}]-4α-4_6S9 in the presence of DPPC vesicles at the various lipid/peptide ratios. (△) [Trp^2]-4α-4_6S9, (○) [Trp^{12}]-4α-4_6S9. [Peptide] = 5 μM. The fluorescence intensity was measured at 25°C, 5 min after the addition of the peptides. The emission wavelength was 280 nm.

CONCLUSIONS

Hydrophobicity and helicity have more influence on membrane perturbation than membrane fusion, because increase in the Ser residue content in the bundled peptides caused a decrease in their perturbation activity (Figure 4), whereas this increase caused little change in their fusion activity (Figure 5). Furthermore, the fusion can be said not to coincide with the membrane perturbation, because it took place at a lower lipid/peptide ratio [29]. The hydrophobicity/hydrophilicity balance is important for the design of lipid membrane-interacting peptides.

The results of this study, which were different from the case of[Trp^1]- and [Trp^{12}]-4α-4_6S, suggested that [Trp^2]- and [Trp^{12}]-4α-4_6S9 lie on the surface of lipid membranes. In this situation, the hydrophobic region of the peptides might be slightly embedded under the surface of the membrane, because membrane perturbation and fusion activities of the peptides were observed. The behavior of bundled peptides was found to be again much different from that of the corresponding monomer peptides on interaction with the membrane, suggesting that other interesting and unique properties may be derived by the bundling of peptides.

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