Enhanced Membrane-Perturbing Activities of Bundled Amphiphilic α-Helix Polypeptides on Interaction with Phospholipid Bilayer

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The 24-peptide **4$_6$**, designed to take an amphiphilic α-helix structure, had strong activity toward phospholipid membranes and could form ion-channels selective for cations on a planar membrane [*J. Biol. Chem.*, **266**, 20218 (1991)]. Four, six, and eight segments of the peptide **4$_6$** were bundled (**4α-4$_6$**, **6α-4$_6$**, and **8α-4$_6$**) on dendrimers consisting of Lys residues. These bundled peptides had highly α-helical structures with an enhanced α-helicity compared with the original **4$_6$** peptide. These peptides had hydrophobic pockets inside the bundle structures in aqueous solution in which the fluorescent hydrophobic probe, 1-anilino-8-naphthalenesulfonate (ANS), bound strongly. The peptides caused much leakage of carboxyfluorescein from small unilamellar vesicles of phospholipids at much lower concentrations of peptides than **4$_6$** did. Furthermore, the peptides induced the vesicle fusion at lower concentrations than those of the leakage. Four segments of the analogous 24-peptide **4$_6$S**, in which six Ser residues were introduced instead of Ala and Leu residues in **4$_6$**, were bundled by the same method (**4α-4$_6$S**). The fluorescent properties of the Trp residues incorporated at the 1 and 12 positions in **4α-4$_6$S**, respectively, indicated that the centers of the helices were in more hydrophobic conditions than the *N*-terminal was. These facts caused us to conclude that the bundled conformation increased the perturbation of the phospholipid membrane, and as a result, the peptides were embedded in the membrane.

Much attention has been attracted to the properties of amphiphilic α-helix peptides,[1,2] for example, membrane perturbation,[3—10] membrane fusion,[4] cell lysis,[8—10] antimicrobial activity,[3,5,9,10] and ion-channel formation.[5,6,11—13] On the basis of the relationship between the conformation and the activities discussed, these functions are generally attributed to the amphiphilic characteristics of the secondary structure of polypeptides. On the other hand, the bundled α-helical peptides and proteins have been extensively designed and synthesized with single-chain polypeptides,[14—16] a template-assisted approach,[3,17,18] and oligoLys connection.[19,20] These designed peptides and proteins were also studied to examine the influences on conformational stability and property,[14—17] catalytic activity,[18,19] and ion-channel formation.[13] However, other activities of the bundled peptides on the membrane, i.e., perturbation, fusion, and lysis, have rarely been studied[9] despite the intrinsic activity of amphiphilic α-helix peptides. To know how the bundling of amphiphilic α-helix peptides influences such membrane activities would be significant for design of artificial proteins, especially with ion-channel activity.

The designed peptide **4$_6$** has an amphiphilic α-helix structure, and causes dye to leak from phospholipid vesicles.[5a] Furthermore, the peptide forms ion-channels in planar membranes that are selective for cations. The mechanism of the ion transport was proposed to be that the aggregation of the amphiphilic peptide made a hole the inside of which was hydrophilic. However, such an aggregated form could not be identified in our previous work.[5,6] Therefore, we attempted to synthesize polypeptides with several helices gathered in the same molecule to detect the aggregated number responsible for the ion-transport. Here, we report the synthesis of the bundled polypeptides, **4α-4$_6$**, **6α-4$_6$**, and **8α-4$_6$**, which have 4, 6, and 8 segments of the **4$_6$** chain (Fig. 1) and their properties in the interaction with phospholipid bilayer membranes. These bundled polypeptides had highly increased activities to disturb and to fuse

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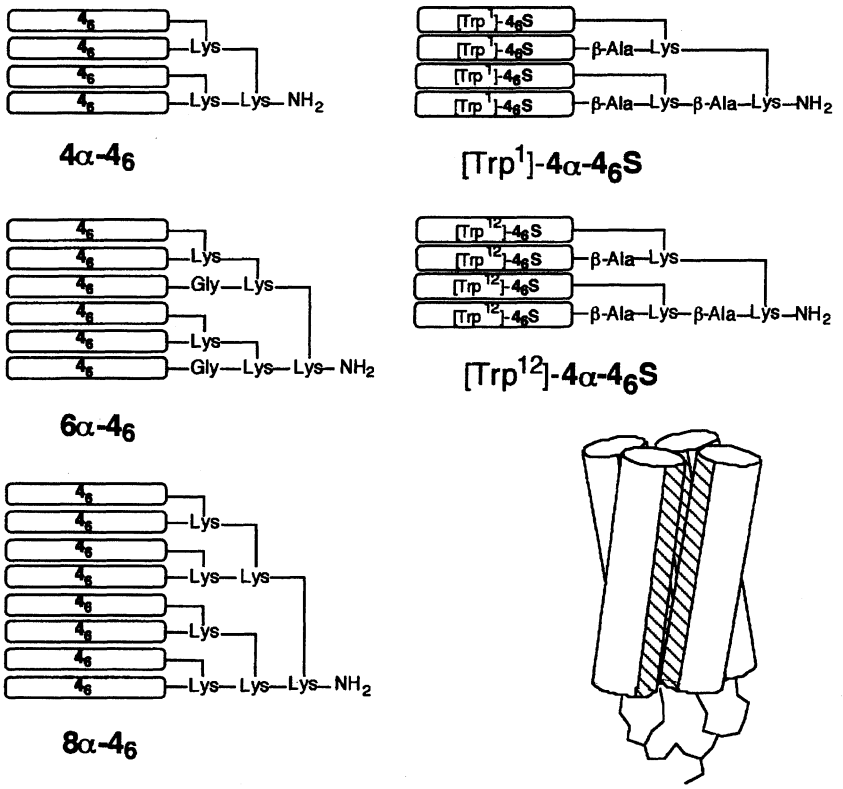

4$_6$: H-(Leu-Ala-Arg-Leu)$_3$-(Leu-Arg-Ala-Leu)$_3$-

4$_6$S: H-(Leu-Ala-Arg-Ser)$_2$-Leu-Ser-Arg-Leu-
Leu-Arg-Ser-Leu-(Ser-Arg-Ala-Leu)$_2$-

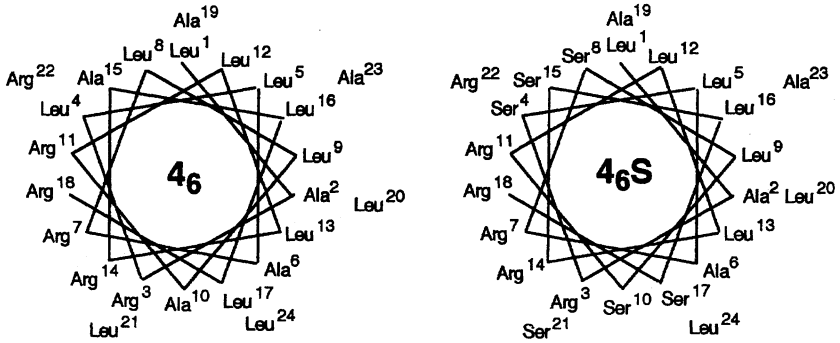

Fig. 1. Structure of the amphiphilic α-helix bundle peptides, and illustrations of α-helical wheels and 4α-helix bundle structure.

the membranes. The former activity was also observed in the experiment of electron current measurements in a planar membrane. Moreover, we synthesized another polypeptide, **4α-4$_6$S**, in which four chains of the 24-peptide **4$_6$S** were bundled (Fig. 1). The 24-peptide **4$_6$S** was designed to have an amphiphilic α-helix structure with six Ser residues instead of Ala and Leu residues in **4$_6$**. Therefore, the peptide was known to have weakened activity of membrane perturbation due to its reduced hydrophobicity.[5b] The introduced Trp residues in **4α-4$_6$S** gave information on the interaction mode of the peptide with membranes.

Results and Discussion

Peptide Design and Synthesis. Peptides were designed to form bundle structures of α-helices consisting of 4, 6, and 8 chains of segments on dendrimer-type cores composed of Lys residues (Fig. 1), which were originally introduced as supports for the multiple antigenic peptides (MAP).[20] The 24-peptide segment **4$_6$** was previously designed to take an amphiphilic α-helix structure using a couple of the 3 times-repeating

tetrapeptides, Leu–Ala–Arg–Leu and Leu–Arg–Ala–Leu [(Leu–Ala–Arg–Leu)$_3$–(Leu–Arg–Ala–Leu)$_3$].[5] The related 24-peptide **4$_6$S** was designed on the basis of the **4$_6$** sequence to reduce the hydrophobicity of the peptide using six hydrophilic Ser residues instead of hydrophobic Leu and Ala residues. As a consequence, the hydrophilic region of the helix was increased from 1/4 in **4$_6$** to 1/2 in **4$_6$S** (Fig. 1). For the latter case, a Trp residue was introduced at the *N*-terminal or the center of the helix to monitor the peptide-phospholipid interaction. The structure of the Lys dendrimer was modified with a flexible anchor of β-Ala residues.

Synthesis was done by the solid-phase method on MBHA[21] resin. The substitution level of the first amino acid was reduced (0.1 mmol/g resin) to increase the coupling efficiency of Boc–amino acids on the Lys core. The peptides synthesized were highly soluble in aqueous solution in spite of their high molecular weights. They were eluted with proportional mobility to their molecular weights on gel filtration chromatography with Sephadex G-50 (10% AcOH), indicating that the peptides had the expected molecular weights and they were in a monomeric form under the conditions.

CD Study. CD spectra of the bundled peptides were measured in aqueous solution, TFE, and in the presence of various phospholipid vesicles (DPPC, DPPC/DPPG (3/1), and EYPC) (Fig. 2). All the peptides showed typical α-helical CD patterns, especially, **4α-4$_6$**, **6α-4$_6$**, and **8α-4$_6$** took almost complete α-helical structures (>80%). The peptides, [Trp^1]-**4α-4$_6$S** and [Trp^{12}]-**4α-4$_6$S**, with reduced hydrophobicity showed lower α-helicity than the **4$_6$** series. The α-helical contents of these peptides are summarized in Table 1.[22] The α-helicities of both series of the peptides were increased compared with those original peptides, **4$_6$** and **4$_6$S**, in aqueous solution. These results suggested that the α-helical segments formed bundle structures, which were stabilized with the interaction between the hydrophobic faces of the amphiphilic helices.[1,2,14—18] This suggestion was also confirmed by the ratio of the ellipticities at two maxima, $[\theta]_{222/208}$, being close to 1.0 (ca. 0.90) in the buffer, indicating the presence of he-

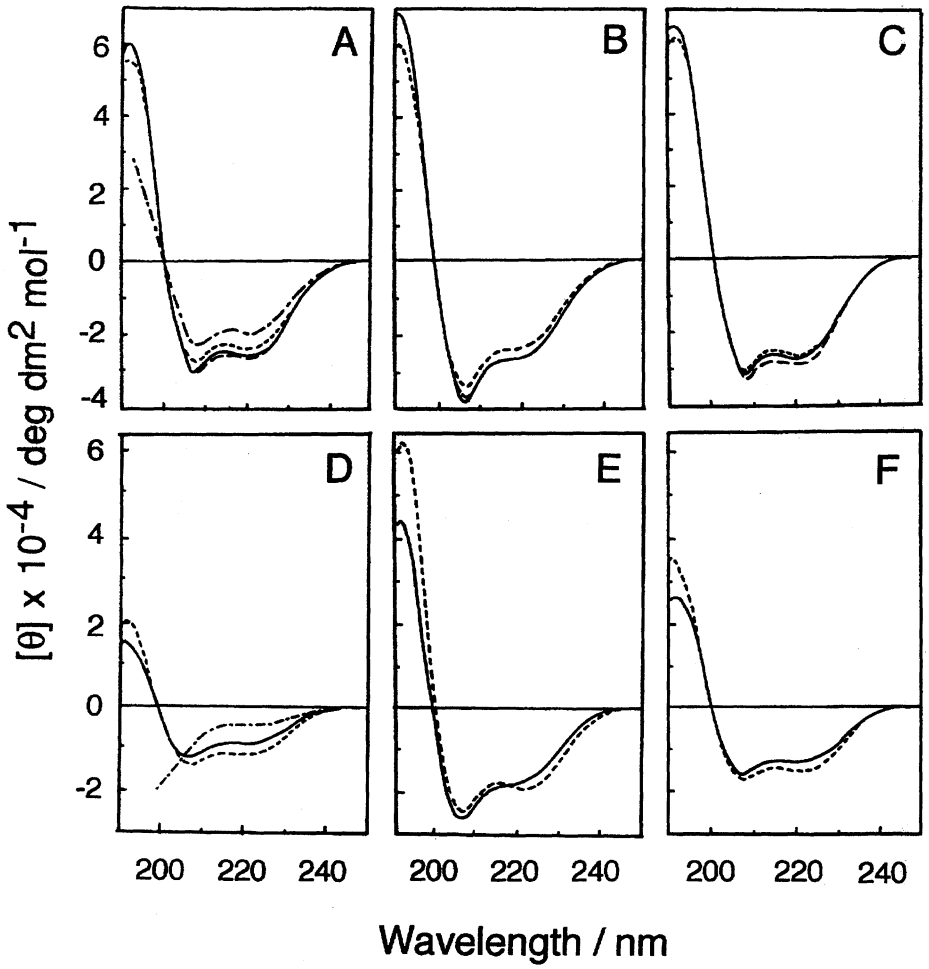

Fig. 2. CD spectra of the peptides under various conditions. A and D, 2.0×10^{-2} mol dm^{-3} Tris HCl buffer (pH 7.4); B and E, TFE; C and F, DPPC vesicles (0.90×10^{-3} mol dm^{-3}). (—) **4α-4$_6$** (A, B, C) and [Trp^1]-**4α-4$_6$S** (D, E, F); (---) **6α-4$_6$** (A, B, C), and [Trp^{12}]-**4α-4$_6$S** (D, E, F); (– – –) **8α-4$_6$** (A, B, C); (–·–·–·–) **4$_6$** (A) and **4$_6$S** (D). [Peptide]$=5.0\times10^{-6}$ mol dm^{-3}; 25 °C.

Table 1. α-Helix Contents of the Peptides under Various Conditions

Peptide	α-Helix content/%							
	Buffer		TFE	DPPC		DPPC/DPPG		EYPC
	25 °C	50 °C	25 °C	25 °C	50 °C	25 °C	50 °C	25 °C
4α-4$_6$	83	82	85	89	95	n.d.[a]	n.d.[a]	90
6α-4$_6$	77	74	75	86	81	n.d.[a]	n.d.[a]	91
8α-4$_6$	85	90	85	95	95	n.d.[a]	n.d.[a]	99
[Trp^1]-**4α-4$_6$S**	30	18	57	51	40	n.d.[a]	n.d.[a]	—[b]
[Trp^{12}]-**4α-4$_6$S**	38	22	64	51	45	n.d.[a]	n.d.[a]	—[b]
4$_6$	65	—[b]	—[b]	60	—[b]	60	—[b]	—[b]
4$_6$S	15	22	94	70	70	n.d.[a]	98	86

a) Correct values could not be determined. b) Not carried out.

lix–helix interactions, but it was ca. 0.70 in TFE.[23] In TFE, the α-helicities were still high (**4$_6$** series) and increased (**4$_6$S** series). In this condition, α-helical segments were apart from each other, but the helix conformation was stabilized by the effect of the solvent.[16] Moreover, the α-helical structures were increased by the addition of phospholipid vesicles, suggesting that the peptides could interact with the phospholipid bilayer membranes examined. The ratio of $[\theta]_{222/208}$ was approximately 0.9, indicating that the existence of helix–helix interaction forming a bundle structure in the lipid environments. In the case of DPPC/DPPG vesicles, the peptides took highly α-helical structures but the spectra could not be correctly measured, because the peptides caused extreme aggregations of the vesicles. The CD measurements at 50 °C showed that the α-helicity was not significantly changed in the absence or presence of vesicles. This indicated that the α-helical structures were very stable.

Fluorescence Study. To obtain information on the tertiary structure of the bundled peptides, the binding properties of the hydrophobic probe, 1-anilino-8-naphthalenesulfonate (ANS), to the hydrophobic pockets in the bundle peptides were examined in aqueous solution. With increasing concentrations of the peptides, the intensities of ANS fluorescence were increased (Fig. 3). These results indicated that the peptides were bundled to form hydrophobic pockets inside the structures in aqueous solution. The binding constants calculated from the titration were 6.0×10^4, 17×10^4, and 22×10^4 mol^{-1} dm^3, respectively, for **4α-4$_6$**, **6α-4$_6$**, and **8α-4$_6$**, assuming the 1 : 1 complex formation with ANS. The dye ANS bound more strongly with increasing the chain number. These values are comparable to those of designed 4α-helix bundle proteins (2.0×10^4 and 2.1×10^4 mol^{-1} dm^3)[14a,14b,15] and native proteins, apomyoglobin (12×10^4 mol^{-1} dm^3)[14b] and β-lactamase (2.5×10^4 mol^{-1} dm^3).[24] The binding properties of ANS to the peptides [Trp^1]-**4α-4$_6$S** and [Trp^{12}]-**4α-4$_6$S** were similar to those of the **4$_6$**-series peptides (data not shown). The binding constants were 2.3 and 3.6×10^4 mol^{-1} dm^3, respectively, for [Trp^1]-**4α-4$_6$S** and [Trp^{12}]-**4α-4$_6$S**. The **4α-4$_6$S** peptides with narrower hydrophobic region had lower binding ability of ANS than the **4$_6$** series.

The maximum wavelength of the ANS fluorescence

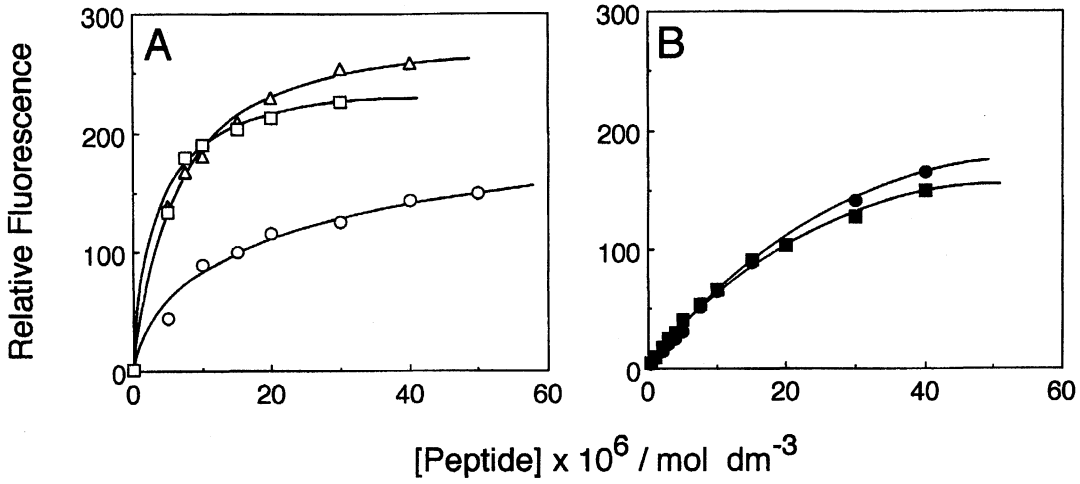

Fig. 3. Dependence of the fluorescence intensity of ANS on the peptide concentration. A, (○) **4α-4$_6$**; (△) **6α-4$_6$**; (□) **8α-4$_6$**. B, (●) [Trp^1]-**4α-4$_6$S**; (■) [Trp^{12}]-**4α-4$_6$S**. [ANS]$=2.0\times10^{-6}$ mol dm^{-3}; excited at 345 nm; 25 °C in the buffer.

shifted from 515 to 460 nm by the addition of the peptides. Those at the saturated state were 460, 459, and 462 nm, respectively, for **4α-4$_6$**, **6α-4$_6$**, and **8α-4$_6$**, indicating that ANS bound in a similar environment to pentanol (λ_{max}=460 nm).[14b] The λ_{max} of the dye bound in native protein, apomyoglobin, and proteins in molten-globule states were 454 nm and 470—490 nm,[14b,24] respectively. It is interesting that the λ_{max} of the designed bundle peptides were between those of native proteins and molten globules. These results are comparable to those by DeGrado et al.[14b] and Morii et al.[15] The fluorescent behaviors of dodecylacridine orange with the peptides were similar to the results for ANS (data not shown).

The fluorescent properties of the Trp residues incorporated in the **4α-4$_6$S** peptides were also examined in the absence or presence of DPPC vesicles (Fig. 4). In aqueous solution, the wavelength maxima of [Trp^1]-**4α-4$_6$S** and [Trp^{12}]-**4α-4$_6$S** were 360 and 343 nm, respectively. The Trp residues at the centers of helix segments were in more hydrophobic circumstances than the *N*-terminal ones, also indicating the formation of the bundle structure. With increasing concentrations of phospholipid, the fluorescent maximum was shifted to a longer wavelength (380 nm) in [Trp^1]-**4α-4$_6$S**, whereas it was shifted to a little bit shorter one (338 nm) in [Trp^{12}]-**4α-4$_6$S**. These results suggested that the *N*-terminals of the bundled peptides were in more hydrophilic circumstances than the centers of the helices in the phospholipid bilayer membranes. These findings are not inconsistent with the assumption that the peptides were perpendicularly embedded in the membrane, that is, the *N*-terminals were located around the hydrophilic region of the membrane and the centers of the peptide segments were in the hydrophobic region of the membrane. In this state, the hydrophobic residues in the helices would face outside to the hydrocarbon chains in the membrane. Moreover, the fluorescent properties of the peptides coincide with those of the Trp residues in the single-chain 24-peptide related to **4$_6$S** by Lee et al.[5b] As the results of the peptides interacted with membranes, the fluorescent intensities of the Trp residues of both peptides were decreased by the addition of the vesicles. Though the reduction of the intensities cannot be explained at this point, it may be due to the quenching of the fluorescence by the access of the ion-pair of the lipid head groups to the *N*-terminal Trp, by the close proximity of the Trp residues, and/or by the decrease in mobility of Trp residues into the membrane.

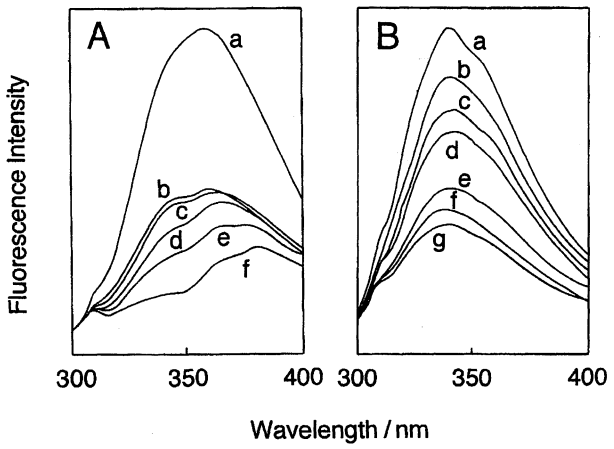

Fig. 4. Fluorescence spectra of [Trp^1]-**4α-4$_6$S** (A) and [Trp^{12}]-**4α-4$_6$S** (B) in the presence of DPPC vesicles at the various lipid/peptide ratios. [DPPC]/[Peptide]; a peptide only, b:1.0, c:2.0, d:4.0, e:8.0, f:20, g:28. [Peptide]=5.0×10^{-6} mol dm^{-3}; excited at 280 nm; 25 °C in the buffer.

Dye Leakage from Vesicles. To study the perturbation by the peptides of phospholipid membranes, leakage of the fluorescent dye, carboxyfluorescein (CF), entrapped inside phospholipid vesicles was examined (Fig. 5).[3—6,25] The peptides caused much dye leakage at very low concentrations of the peptides (<2.0×10^{-7} mol dm^{-3}). Especially, at 50 °C, which is above the phase-transition temperature (42 °C) of DPPC and DPPG, the leakage reached 100%. The natural phospholipid egg yolk lecithin gave similar results (data not shown). Though the fluorescence intensities were measured 2 min after the peptides were added, the maximum leakage occurred quickly, within 30 s. The peptide concentrations reaching the maximum leakage were approximately one or two orders of magnitude less than those of the parent **4$_6$** and **4$_6$S**. When the lipid/peptide ratio was 700, the peptide could cover the surface of the vesicles to 20—40%. The bundling of the amphiphilic α-helices dramatically increased the membrane-perturbation activity. At 25 °C, **4$_6$** and **4$_6$S** did not have strong perturbation activity, but the bundling could generate the activity. The order of the activity was **8α-4$_6$**>**6α-4$_6$**>**4α-4$_6$**>**4α-4$_6$S**, therefore, the activity was increased with increasing the α-helical chain numbers. Although the bundles of the **4$_6$** segment were more effective than those of **4$_6$S**, the **4α-4$_6$S** peptides still had the strong ability. In the case of vesicles containing acidic DPPG, higher activity was expected than that with neutral DPPC because of the increased electrostatic interaction between the cationic peptides and the anionic lipid.[3,4] However, the activity was approximately 1/10 in terms of peptide concentrations. It might be required that the cationic peptides cover the anionic surface of the vesicles to some extent (20—40%) to produce the full activity. These facts suggested that the hydrophobic parts constructed with Leu residues in the helices were more important to the perturbation activity than the electrostatic interaction of the cationic peptides with the anionic lipid.

Membrane Fusion Activity. Fusion of phospholipid bilayers caused by the peptides were evaluated by measurements of intermixing of phospholipids between vesicles containing either NBD-PE or Rh-PE

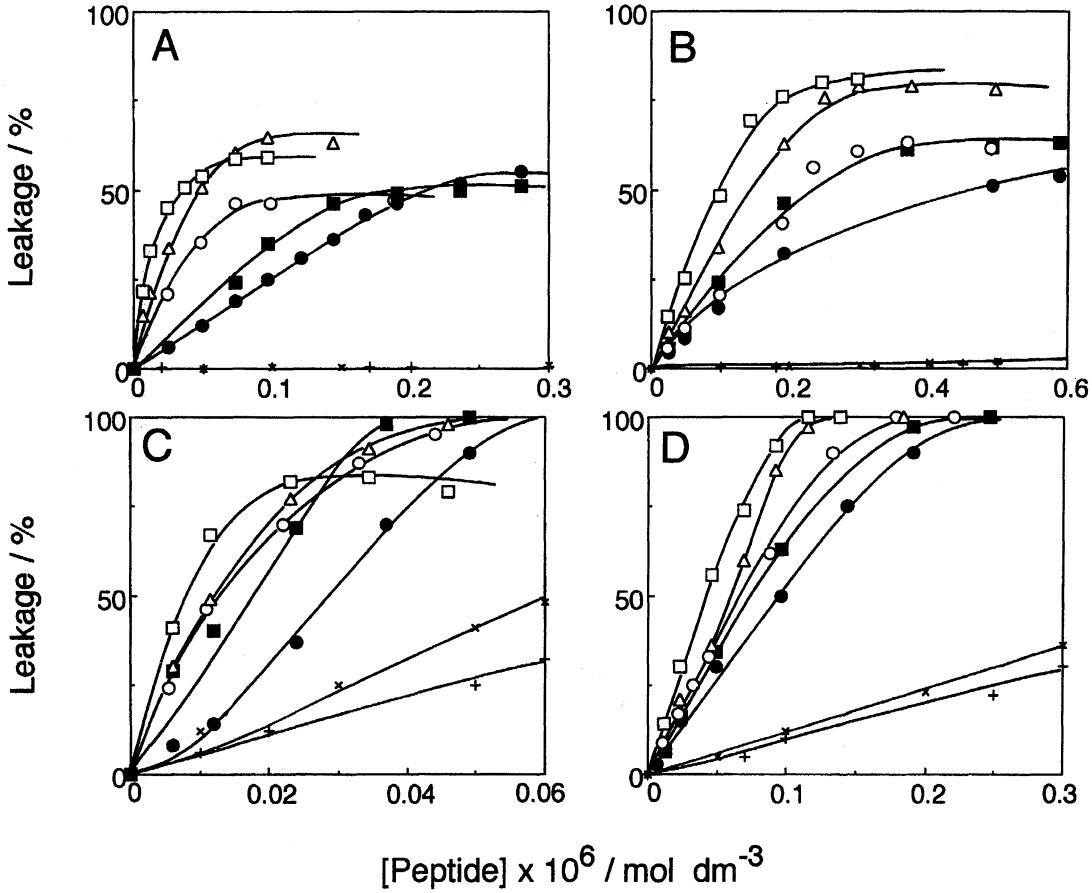

Fig. 5. Profiles of the CF leakage as a function of the peptide concentration. A, DPPC at 25 °C; B, DPPC/DPPG (3/1) at 25 °C; C, DPPC at 50 °C; D, DPPC/CPPG (3/1) at 50 °C. (○) **4α-4$_6$**; (△) **6α-4$_6$**; (□) **8α-4$_6$**; (●) [Trp^1]-**4α-4$_6$S**; (■) [Trp^{12}]-**4α-4$_6$S**; (×) **4$_6$**; (+) **4$_6$S**. [Lipid]=7.0×10^{-5} mol dm^{-3} in the buffer.

(Fig. 6).[4,26] The intermixing of the chromophoric phospholipids quenched NBD fluorescence at 530 nm by the energy transfer to rhodamine. The peptides caused the fusion of the DPPC vesicles almost completely at peptide concentrations of 0.02—0.04×10^{-6} mol dm^{-3}, which were one order less than those required for the

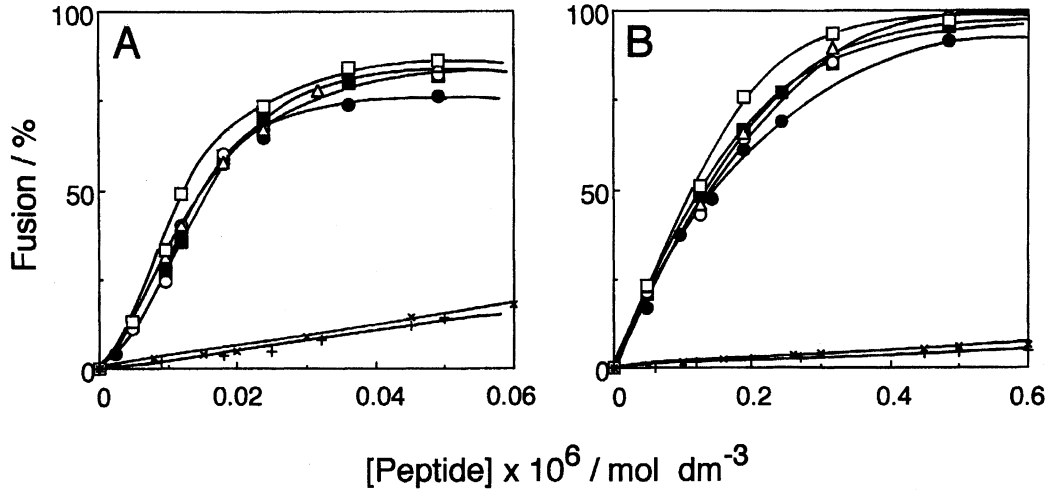

Fig. 6. Profiles of the vesicle fusion as a function of the peptide concentration. A, DPPC at 25 °C; B, DPPC/DPPG (3/1) at 25 °C. (○) **4α-4$_6$**; (△) **6α-4$_6$**; (□) **8α-4$_6$**; (●) [Trp^1]-**4α-4$_6$S**; (■) [Trp^{12}]-**4α-4$_6$S**; (×) **4$_6$**; (+) **4$_6$S**. [Lipid]=7.0×10^{-5} mol dm^{-3} in the buffer.

leakage activity at 25 °C. It is worth noting that the bundled peptides **4α-4$_6$S** had almost the same fusion ability as the bundled peptides of **4$_6$**. Furthermore, the peptide concentrations required for the maximum fusion of DPPC vesicles were 1/10 of those with DPPC/DPPG vesicles. The concentrations are comparable with those in the leakage activity of the peptides for DPPC/DPPG. It could be considered that the hydrophobic interaction might be more important than the electrostatic interaction between cationic peptide and anionic vesicles, but this interpretation does not explain the unexpected high activity of **4α-4$_6$S**. These results also indicated that the fusion incident did not coincide with the perturbation of the phospholipid vesicles, and the former took place at a lower lipid/peptide ratio.

Planar Membrane Current. The current across the planar membrane of EYPE/EYPC (3/1) was measured with **4α-4$_6$** (13×10^{-9} mol dm^{-3}), **6α-4$_6$** (9.3×10^{-9} mol dm^{-3}), and **8α-4$_6$** (6.7×10^{-9} mol dm^{-3}) by applying various membrane potentials. No peptides showed the formation of stable ion channels, but they caused current increases with an erratic conductance level (Fig. 7). The effect was enhanced with increasing numbers of helices. This was comparable to the activity of membrane perturbation. Therefore, the erratic current flow might occur as a result of the membrane perturbation by the peptides.

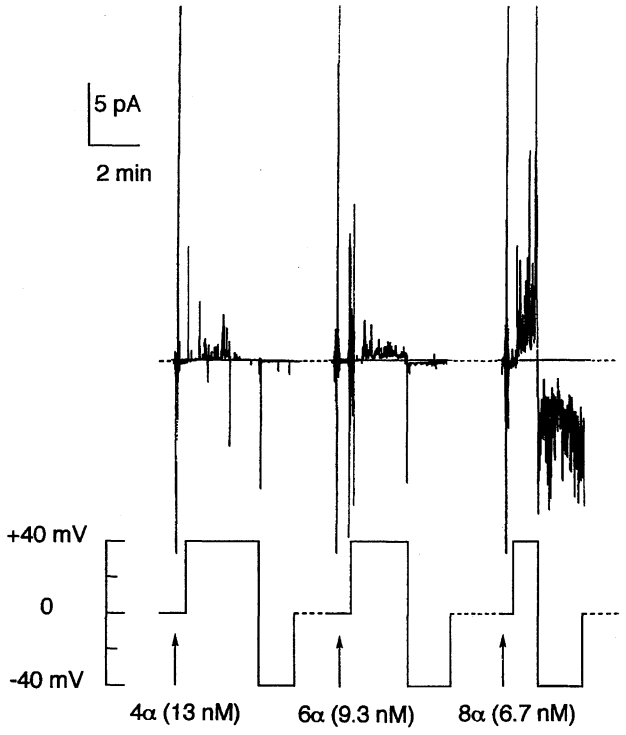

Fig. 7. Ionic current measurements of **4α-4$_6$**, **6α-4$_6$**, and **8α-4$_6$**. The membrane potentials indicated were applied from *cis* to *trans* compartments. Peptide concentrations are also shown in this figure.

Antimicrobial Activity. None of the peptides showed the growth inhibitory activity at peptide concentrations of less than 100 μg ml^{-1} against various strains of *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli*. The related single chain peptides **4$_3$** (12 residues)[3] and **4$_6$** (24 residues)[5] had strong (3—6 μg ml^{-1}) or substantial (50 μg ml^{-1}) activity, respectively, against Gram-positive bacteria. The antimicrobial activity did not directly correlate with the perturbation and fusion activity of the model phospholipid membranes. It would be necessary for the activity that a peptide could easily path through cell walls to reach the membranes, that is, peptides with large molecular weight might not reach the cell membranes.

Experimental

Reagents used for the synthesis and analysis were of reagent grade. Peptide synthesis was done manually by the Boc strategy in a glass vessel. Amino acid derivatives and the resin were purchased from Watanabe Chemical (Hiroshima). Carboxyfluorescein from Kodak was purified with recrystallization as described in the literature.[4] DPPC, DPPG, and EYPC were purchased from Sigma, the NBD-PE and Rh-PE were from Avanti Polar Lipids. Fluorescent data were taken on a Hitachi spectrofluorometer F-3010 with a thermostatted cell holder. Amino acid analyses were done by the ninhydrin method on a JEOL JLC-300 analyzer after hydrolysis of the peptides at 110 °C for 24 h in 6.0 mol dm^{-3} HCl.

Peptides. General Methods. Peptides were synthesized by the stepwise elongation of Boc–amino acid on MBHA resin (0.68 mmol amine/g resin). The Boc–amino acid derivatives used were as follows: Ala, Arg(Tos), β-Ala, Gly, Leu, Lys(Boc), Ser(Bzl), and Trp(HCO). Fmoc-Lys(Boc) was also used to prepare a Lys dendrimer part as described below. The first amino acid (0.40 mmol) was reacted with the resin (1.0 g) by the BOP–HOBt method[27] to give an amino acid resin with a rather low substituted level (0.10 mmol g^{-1}). All the peptides were synthesized starting from a Boc–amino acid resin (0.50 g, 0.05 mmol). The coupling protocol was the following: (1) wash, DCM (×2), (2) wash, 50% TFA/DCM (×1), (3) deprotection, 50% TFA/DCM (20 min), (3) wash, DCM (×4), (4) wash, 5% DIEA/DCM (×4), (5) wash, DCM (×3), (6) wash, DMF (×1), (7) coupling Boc–amino acid (4 molar amounts), BOP (4 molar amounts), HOBt (4 molar amounts), and DIEA (6 molar amounts) (60 min), (8) wash, DMF (×3), (9) wash, DCM (×2), and (10) Kaiser Test.[28] When the coupling was incomplete, the protocol was repeated from (6). The protecting groups and the resin were removed with anhydrous HF (20 ml) in the presence of anisole (1 ml) at 0 °C for 90 min. When the peptides contained Trp(HCO) residues, the formyl groups were deprotected with the treatment of peptides in 0.1 mol dm^{-3} NaOH at 0 °C for 5 min. The removal of the protecting groups was checked by the UV spectra. The crude peptides were passed through a column of Sephadex G-50 (2.5×90 cm) with 10% AcOH several times to give a sharp peak on the chromatography. Peptides gave satisfactory results on amino acid analyses.

4α-4$_6$·28AcOH. Boc–Lys(Boc)–OH was introduced

onto MBHA resin by the BOP–HOBt method as described above. The Boc–Lys(Boc)–Lys[Boc–Lys(Boc)]–MBHA resin was synthesized from the Boc–Lys(Boc)–resin and Boc–Lys(Boc)–OH by the coupling protocol. The stepwise elongation of Boc–amino acids was done by the same method. The protecting groups and the resin were removed with anhydrous HF. The crude peptide was purified by gel filtration as described above. Yield 160 mg (25%). Amino acid analysis: Ala(24) 24.0, Leu(48) 47.7, Lys(3) 3.3, Arg(24) 24.0.

6α-4$_6$·42AcOH. To the Boc–Lys(Boc)–MBHA resin was introduced Boc–Gly–Lys[Boc–Lys(Boc)]–OH, which was synthesized by the solution method using *N*,*N*-dicyclohexylcarbodiimide and HOBt at 0 °C[29] and identified with FAB-MS, *m*/*z* 632 $(M+H)^+$, to give the Boc–Gly–Lys[Boc–Lys(Boc)]Lys{Boc–Gly–Lys[Boc–Lys(Boc)]}-resin. The stepwise elongation of Boc–amino acids, the deprotection, and the purification were done by the method described above. Yield 150 mg (16%). Amino acid analysis: Gly(2) 2.2, Ala(36) 36.0, Leu(72) 73.4, Lys(5) 5.4, Arg(36) 34.9.

8α-4$_6$·56AcOH. To the Boc–Lys(Boc)–Lys[Boc– Lys(Boc)]–MBHA resin prepared for **4α-4$_6$** was introduced Boc–Lys(Boc)–OH to give the Boc–Lys(Boc)–Lys[Boc–Lys(Boc)]–Lys{Boc–Lys(Boc)–Lys[Boc–Lys(Boc)]}-resin. The stepwise elongation of Boc-amino acids, the deprotection, and the purification were done by the method described above. Yield 330 mg (27%). Amino acid analysis: Ala(48) 48.0, Leu(96) 92.4, Lys(7) 7.6, Arg(48) 46.8.

[Trp^1]-4α-4$_6$S·28AcOH. Fmoc–Lys(Boc)–OH was introduced to the MBHA resin by the BOP–HOBt method. After the Fmoc group was removed with 20% piperidine/DMF for 15 min, Boc–β-Ala–OH was coupled by the same method. To the Boc–β-Ala–Lys(Boc)-resin were introduced Fmoc–Lys(Boc)–OH and Boc–β-Ala–OH in this order to give the Boc–β-Ala–Lys(Boc)–β-Ala–Lys[Boc–β-Ala–Lys(Boc)]-resin. The stepwise elongation of Boc–amino acids and the deprotection were done by the method described above. The formyl groups of Trp residues were deprotected with the treatment of peptides in 0.1 mol dm^{-3} NaOH at 0 °C for 5 min. The crude peptides were purified by gel filtration as described above. Yield 95 mg (15%). Amino acid analysis: Ser(24) 22.8, Ala(16) 16.0, Leu(28) 29.5, Lys(3) 3.2, Arg(24) 26.0.

[Trp^{12}]-4α-4$_6$S·28AcOH. [Trp^{12}]-**4α-4$_6$S** was synthesized by the same method as described for [Trp^1]-**4α-4$_6$S** from the Boc–β-Ala–Lys(Boc)–β-Ala–Lys[Boc–β-Ala–Lys(Boc)]-resin. The deprotection and the purification were done by the method described above. Yield 110 mg (17%). Amino acid analysis: Ser(24) 22.5, Ala(16) 16.0, Leu(28) 30.0, Lys(3) 3.3, Arg(24) 23.5.

The results of the gel filtration of the peptides on Sephadex G-50 (2.5×90 cm) with 10% AcOH gave information on the ratio of molecular weights of the peptides. The semilogarithmic plots of the molecular weights of the peptides as a function of the retention volumes showed a linear correlation, indicating that the peptides had almost the correct molecular weights and they are in a monomeric state in the solvent. Retention volume/ml (molecular weight): **4α-4$_6$**, 225 (11300); **6α-4$_6$**, 200 (17100); **8α-4$_6$**, 190 (22700); [Trp^1]-**4α-4$_6$S**, 220 (11100); [Trp^{12}]-**4α-4$_6$S**, 220 (11100).

Preparation of Phospholipid Vesicles. Small unilamellar vesicles of DPPC, DPPC/DPPG (3/1), and EYPC were prepared for CD, fluorescence, leakage, and fusion experiments as follows.[5,6] Phospholipid (7.5 mg) was dissolved in $CHCl_3$ and MeOH (2 ml) and then dried by a stream of nitrogen gas. The dried lipid was hydrated in 10 ml of 2.0×10^{-2} mol dm^{-3} Tris HCl buffer (pH 7.4) using a Branson bath-type sonicator. The suspension was sonicated at 50 °C for DPPC and DPPG, and 25 °C for EYPC for 20 min under a nitrogen atmosphere using a Taitec Ultrasonic Processor VP-5T at 10 W intensity. The vesicles were left for 30 min at 25 °C for DPPC and DPPG, and 0 °C for EYPC before using the measurements. Lipid concentration was 1.0×10^{-3} mol dm^{-3}.

The vesicles trapping CF were prepared by the reported procedure.[5,6] The dried lipid (20 mg) was hydrated in 2.0 ml of 2.0×10^{-2} mol dm^{-3} Tris HCl buffer (pH 7.4) containing 0.10 mol dm^{-3} carboxyfluorescein. The CF-entrapped vesicles were separated from free CF by gel filtration using Sephadex G-75 (1.0×20 cm) with the same buffer (pH 7.4).

CD Measurements. 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 5.0×10^{-6} mol dm^{-3} in solution or in the presence of phospholipid vesicles (0.90×10^{-3} mol dm^{-3}). Measurements were done at 25 or 50 °C. The CD data were evaluated by the method by Chen et al.[22]

CF Leakage. The dye-leakage experiment was done by the procedure of Weinstein et al.[25] and Lee et al.[3—6] To a 2.0 ml of 2.0×10^{-2} mol dm^{-3} Tris HCl buffer (pH 7.4) in a cuvette was added 20 μl of the vesicles containing 0.10 mol dm^{-3} CF to give a vesicle solution with a final concentration of 7.0×10^{-5} mol dm^{-3} lipid. 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 measured 2 min after the addition of the peptides. To measure the fluorescence intensity for 100% dye release, 10 μl of Triton® X-100 (20% 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, F_0 and F_t are intensities observed without the peptides and after Triton® X-100 treatment, respectively.

Fusion Assay.[4,26] Two kinds of vesicles containing NBD-PE and Rh-PE (2.0 mol% each) were prepared by sonication as described above. Equimolar amounts of the vesicles were mixed at 25 °C and the final concentration of lipids was adjusted to 7.0×10^{-5} mol dm^{-3}. The peptides were added to the mixed vesicles and the decrease in NBD fluorescence was recorded continuously at an excitation wavelength of 450 nm and an emission wavelength of 530 nm. 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 pepetides.

Current Measurements on a Planar Membrane. The planar bilayer of EYPE/EYPC (3/1) was prepared by the folding method and the current was measured with Ag/AgCl electrodes as reported previously.[5,6]

Antimicrobial Assay. The minimum inhibitory concentration was measured by the standard agar dilution

method as described previously.[6c]

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21) Abbreviations used were as follows: ANS, 1-anilino-8-naphthalenesulfonate; Boc, *t*-butyloxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; Bzl, benzyl; CF, carboxyfluorescein; DCM, dichloromethane; DIEA, ethyldiisopropylamine; DMF, *N,N*-dimethylformamide; DPPC, dipalmitoyl-DL-3-phosphatidylcholine; DPPG, dipalmitoyl-DL-3-phosphatidylglycerol; EYPC, egg yolk phosphatidylcholine; EYPE, egg yolk phosphatidylethanolamine; HOBt, 1-hydroxybenzotriazole; MBHA, 4-methylbenzhydrylamine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-3-phosphatidylethanolamine;Rh-PE, *N*-[(lissamine rhodamine B)-4-sulfonyl]-dipalmitoyl-3-phosphatidylethanolamine; TFE, 2,2,2-trifluoroethanol; Tos, toluenesulfonyl.

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