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Relationship between antimicrobial activity and amphiphilic property of basic model peptides

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Several cationic model peptides of the prepiece moieties of mitochondrial protein precursors were found to be active against Gram-positive bacteria, but inactive against Gram-negative bacteria. The CD spectra of the model peptides in the presence of phospholipid liposomes demonstrated that antimicrobial activity was generally in parallel with the content of the α-helical amphiphilicity. The results indicate that appropriate positioning of cationic and hydrophobic groups in the stable α-helical structure of the peptides is important to exhibit antimicrobial activity. These peptides also have an ability to leak carboxyfluorescein from acidic and neutral phospholipid vesicles, suggesting that the peptides interact with the bacterial membrane to perturb it.

Introduction

The prepiece moieties of several mitochondrial protein precursors show a common feature that basic amino acid resudues (arginine and lysine) are distributed between short stretches of hydrophobic amino-acid residues [1–3]. Based on this common characteristic feature found in the prepiece moieties, we synthesized previously several model peptides in which the basic amino-acid residues are distributed periodically by linking sequences of two to four uncharged amino-acid residues (Fig. 1) and their effects on the import and processing of cytochrome *P*-450 (scc) precursor were examined [4]. Some of them predicted to hold α-helical conformation with uncharged residues on one side and charged residues on the other side inhibited strongly the import of the precursor and induced leakage of the enzymes from the inner compartments of mitochondria. Since the synthetic model peptides did not solubilize membrane-bound enzymes from mitochondria, it has been suggested that the peptides interact with membranes in a manner different from that of simple cationic detergents. We further presented evidence that these peptides showed the leaking effect not only on mitochondrial membrane, but also on membranes of microsome, lysosome and erythrocyte [4].

Abbreviations: AcONSu, *N*-acetoxysuccinimide; Boc, *t*-butyloxycarbonyl; DPPC, dipalmitoyl-DL-α-phosphatidylcholine; DPPG, dipalmitoyl-DL-α-phosphatidylglycerol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; Pac, phenacyl; Tos, *p*-toluene-sulfonyl; TFA, trifluoroacetic acid.

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Ac-(Leu-Ala-Arg-Leu)$_1$-NHCH$_3$	4_1
Ac-(Leu-Ala-Arg-Leu)$_2$-NHCH$_3$	4_2
Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$	4_3
Ac-(Leu-Ala-Arg-Leu)$_4$-NHCH$_3$	4_4
Ac-(Ala-Arg-Leu)$_3$-NHCH$_3$	3_3
Ac-(Ala-Arg-Leu)$_4$-NHCH$_3$	3_4
Ac-(Leu-Leu-Ala-Arg-Leu)$_2$-NHCH$_3$	5_2
Ac-(Leu-Leu-Ala-Arg-Leu)$_3$-NHCH$_3$	5_3
Ac-(Leu-Ala-Lys-Leu)$_2$-NHCH$_3$	$4'_2$
Ac-(Leu-Ala-Lys-Leu)$_3$-NHCH$_3$	$4'_3$

Fig. 1. Synthetic model peptides. All amino acids are of the L-configuration.

Recently, amphiphilic antimicrobial peptides including melittin [5], cecropin [6,7] and sarcotoxin [8] have been reported to interact with bacterial membranes. In particular, their biological activities have been found to correlate with the conformations of molecules in biological membranes rather than the molecular conformations in aqueous solution [9]. Similarly to these peptides, gramicidin S, an amphiphilic antimicrobial peptide, interacts not only with bacterial membrane to perturb its structure [10], but also with mitochondrial membrane to uncouple oxidative phosphorylation [11]. We found in a preliminary study that some of the peptides modelled on the prepiece moieties of mitochondrial protein precursors showed antimicrobial activity [12]. This finding prompted us to analyze the secondary structural features of the peptides in phospholipid in order to obtain some insight into the structural feature of the peptide molecule in membrane.

To obtain a better understanding of the structure-function relationship of the antimicrobial peptides, the conformation and the interaction of the model peptides with phospholipid liposomes were studied in the present study by CD spectroscopy and phase-transition release experiments in relation to the antimicrobial activity.

Materials and Methods

Materials. DPPC and DPPG were purchased from Sigma Chemical Co. Carboxyfluorescein from Eastman Kodak Co. was purified twice by recrystallization from ethanol. Sepharose 4B was obtained from Pharmacia Fine Chemicals. All other reagents were of the highest analytical grade.

Synthesis of the peptides. All the peptides were synthesized by the solution method. The synthetic route for Ac-octapeptide-NHCH$_3$ (4_2) is shown in Fig. 2. Two short tetrapeptides were prepared stepwise and then the fragment peptides were coupled by the 1-hydroxybenzotriazole – 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide method [13] to give the protected octapeptide. After acetylation of N-terminal, the Tos group was removed by the HF treatment [14]. Other peptides were synthesized similarly. In the case of lysine-containing peptides, the Z group was used for protection of the ϵ-amino group. The purity of the final products was established by TLC, paper electrophoresis, and elemental and amino-acid analyses. Details of the synthesis are described elsewhere [15].

Assay of antimicrobial activity. We employed the following microorganisms; *Staphylococcus aureus* 1840, *Bacillus subtilis* PCI 219, Escherichia coli O-111, *Klebsiella pneumoniae* DT, *Proteus vulgaris* IFO 3988 and *Shigella flexneri* EW-10. Inhibition of the growth of microorganisms by the model peptides was determined by a dilution method using a trypticase soy agar medium. 5 μl of bacaterial suspension containing 10^7 colony forming units/ml was used for all experiments.

CD spectral measurement. CD spectra were recorded on a JASCO J-40A spectropolarimeter connected with a JASCO data processor Model J-DPY using quartz cell of 1 mm pathlength. Spectra in H_2O and trifluoroethanol were measured at a peptide concentration of 100–400 μM. DPPC vesicles (a mixture of uni- and multilamellar ones) were prepared by sonication for 30 min at 50°C in 20 mM Tris-HCl buffer (pH 7.4), and the peptides were dissolved at a concentration of 10–20 μM in 20 mM Tris-HCl buffer solution containing 0.9 mM DPPC vesicles. All measurements were performed at 20°C. The CD data are expressed as mean residue ellipticities. The per-

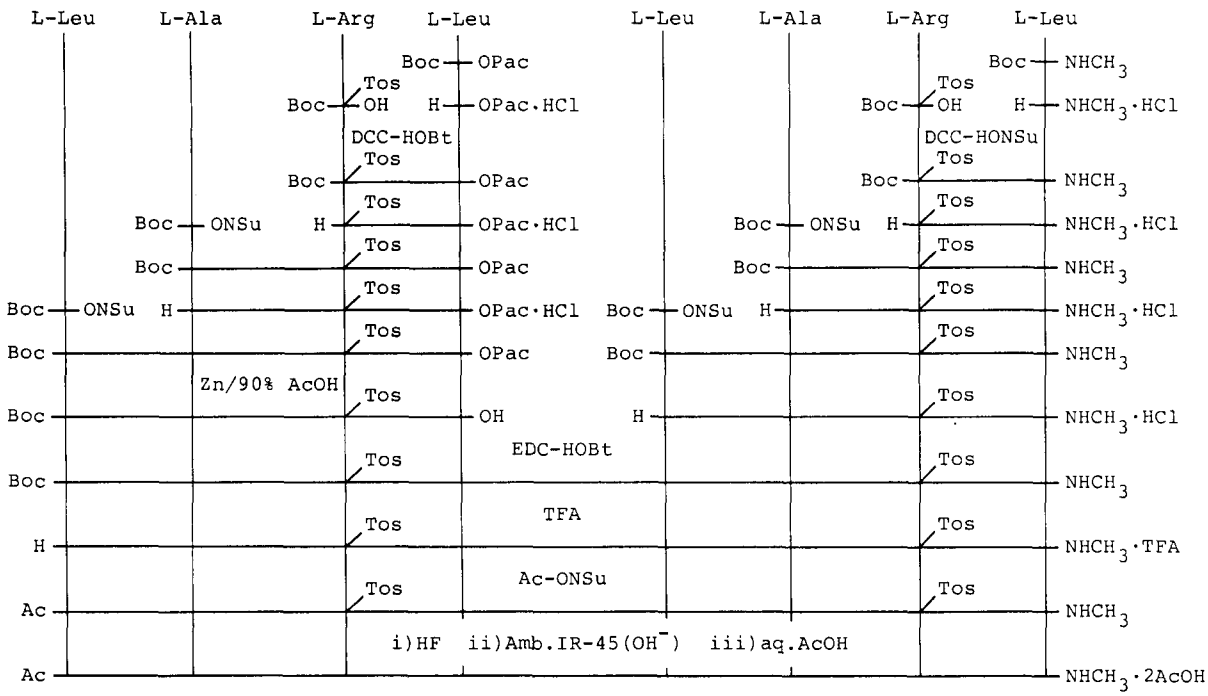

Fig. 2. Synthetic route of Ac-octapeptide-$NHCH_3$ (4_2).

centage of α-helix was calculated on the basis of the theoretical $[\theta]_{220}$ value for a 100% α-helix of the number of amino-acid residues in each peptide as described by Chen et al. [18].

Phase-transition release. The phase-transition release experiment was carried out according to the procedure of Utsumi et al. [16]. Fluorescence spectra were taken with a Hitachi 650-10S Spectrofluorophotometer equipped with a thermostatted cell holder. Carboxyfluorescein was excited at 470 nm and emission at 515 nm was recorded.

DPPC (20 mg) or DPPC-DPPG (20 mg, 3 : 1) was dissolved in 1 ml of chloroform and the solution was dried under a stream of nitrogen in a conical glass tube. The dried lipid (27 μmol) was hydrated in 2.0 ml of 0.1 M NaCl/20 mM phosphate buffer (pH 7.4) containing 100 mM carboxyfluorescein with repeated vortexed-mixing at 50°C for 30 min. The suspension was sonicated at 50°C for 60 min using a Tomy Seiko ultrasonic disruptor Model UR-200P. The mixture of the multilamellar and unilamellar vesicles trapping carboxyfluorescein was subjected to gel-filtration through a Sepharose 4B column (1 × 20 cm) in 0.1 M NaCl/20 mM phosphate buffer (pH 7.4). Fractions (2 ml) were collected and the small unilamellar vesicles collected in fraction 7 were used for phase-transition release measurements.

DPPC or DPPC-DPPG vesicles, all peptides and buffer solution were individually pre-chilled on ice. To 2 ml of 20 mM phosphate buffer (pH 7.4) in the cuvette on ice, was added 50 μl of the vesicles containing 100 mM carboxyfluorescein to give a final concentration of 20 μM phospholipid. 20 μl of an appropriately diluted solution of the peptide in phosphate buffer was added to the mixture. The cuvette was placed in the heated (42°C) cuvette holder of the fluorimeter and both the fluorescence intensity at 515 nm and the temperature were continuously recorded. For determination of the fluorescence intensity derived from 100% dye-release, 10 μl of Triton X-100 solution (20% (v/v) in phosphate buffer) was added to dissolve the vesicles. The percentage of dye release caused by the peptide 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 of post-transi-

tion fluorescence without the peptides and post-Triton X-100 treatment, respectively.

Results

Antimicrobial activity

The minimum inhibitory concentrations, i.e., the minimum amounts of the peptides necessary for complete inhibition of growth of various kinds of bacteria are shown in Table I. It is worth noting that some of the peptides tested showed high antimicrobial activities against Gram-positive bacteria such as *S. aureus* and *B. subtilis*, but not against Gram-negative bacteria such as *E. coli*. The characteristics of the antimicrobial spectra and minimum inhibitory concentrations of the model peptides were similar to those of naturally occurring gramicidin S, which is active against only Gram-positive bacteria and maintains a rigid amphiphilic character. Among the model peptides 4_1–4_4 having the repeating unit (LLeu-LAla-LArg-LLeu), peptide 4_3 showed the highest antimicrobial activity (3 μg/ml) against *B. subtilis*. This potency is exactly the same as that of gramicidin S. The peptides 4_2 and 4_4 had considerable potencies to inhibit the growth of bacteria, while peptide 4_1 was inactive. This indicates that appropriate chain length is important for antimicrobial activity. In this respect, the correlation of activity and chain length of the model peptides tested here differs from that of the linear polypeptides consisting of the repeated pentapeptide sequence of gramicidin S: the antimicrobial activity of the linear peptides increased with an increase of molecular size [17]. When the activities of the peptides 3_3, 4_3, and 5_3, having the same net charges, were compared, peptide 5_3 was 16-times less active than 4_3, and peptide 3_3 was inactive against *B. subtilis*, suggesting that the number of cationic charges is not directly correlated with the antimicrobial activity. No noticeable difference was observed in antimicrobial activity between Arg- and Lys-containing peptides, indicating that difference of cationic character in peptides is not important.

All of these findings support the idea that a definite structure is required for exhibiting the biological activity of the model peptides.

CD study

CD spectra of the peptides 4_2–4_4 in H_2O in comparison with those taken in the presence of liposomes are shown in Fig. 3. In H_2O, the peptide 4_3 exhibited a weak band at 218–222 nm responsible for the α-helix and a strong negative band near at 200 nm responsible for random coil and α-helix. Addition of DPPC led to a marked increase of the band intensity at 218–222 nm and the shift of the band at shorter wavelength to 208 nm, which are due to the increase in the α-helical content. Table II shows α-helical contents in the peptides calculated from the CD spectra at 222 nm under various conditions [18]. Obviously, the peptides except for 4_1 have a moderate ability to form an α-helical structure in an α-helical forming solvent such as trifluoroethanol. On the other hand, in the presence of liposomes there is a difference in their tendency to form an α-helix. Peptides 4_3 and $4_3'$, with the highest antimicrobial activity, showed very high helical contents in the presence of liposomes. Contrary to this, the inactive peptides 4_1 and 3_3 remained in disordered

TABLE I

ANTIMICROBIAL ACTIVITIES OF PEPTIDES

Organism	Minimum inhibitory concentration (μg/ml)										
	4_1	4_2	4_3	4_4	3_3	3_4	5_2	5_3	$4_2'$	$4_3'$	Gramidicin S
S. aureus 1840	>100	25	6.25	25	>100	>100	25	50	50	6.25	3.13
B. subtilis PCI 219	>100	25	3.13	12.5	>100	25	12.5	50	50	3.13	3.13
E. coli O-111	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
S. flexneri EW-10	>100	50	50	>100	>100	>100	>100	>100	50	6.25	6.25
P. vulgaris IFO 3988	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
K. pneumoniae DT	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	25

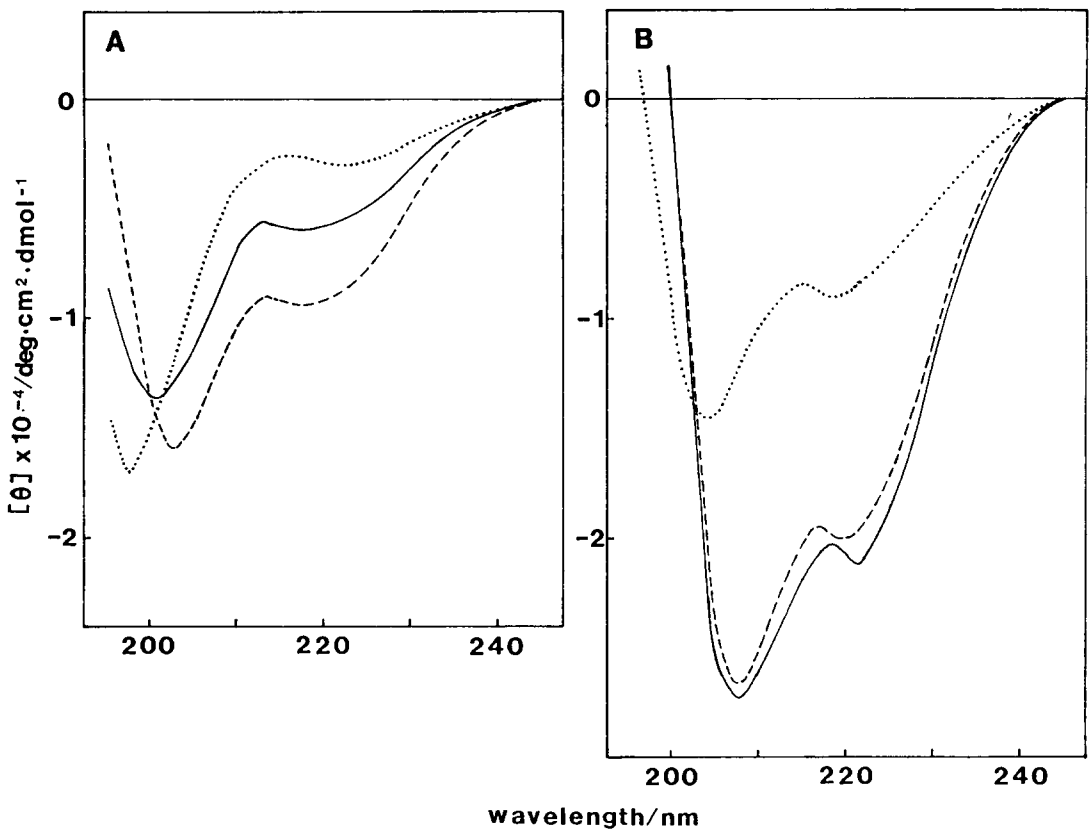

Fig. 3. CD spectra of model peptides 4_2, 4_3, and 4_4 in H_2O (A) and in the presence of DPPC liposomes (B). Curve ·····, 4_2; ————, 4_3; - - - - - -, 4_4.

TABLE II

HELICAL CONTENTS AND EFFECTS ON FLUORESCEIN LEAKAGE OF THE MODEL PEPTIDES AND GRAMICIDIN S

Peptides	α-Helical content (%)			Fluorescein leakage (%) [b]	
	H_2O	trifluoroethanol	liposome [a]	DPPC	DPPC-DPPG
4_1	R [c]	R [c]	R [c]	6	
4_2	R [c]	30	30	30	
4_3	20	35	70	100	95 (50)
4_4	30	40	60	100	
3_3	R [c]	25	R [c]	15	15
3_4	R [c]	35	R [c]	60	100 (70)
5_2	20	40	50	100	100 (95)
5_3	20	35	35	100	100 (90)
$4_2'$	R [c]	30	30	45	
$4_3'$	20	40	50	100	100 (65)
Gramicidin S	–	–	–	80	

[a] In the presence of DPPC vesicles as described in Materials and Methods.

[b] Values are intensities of fluorescence at scanning time of 5 min. Concentrations of peptides are 1 μg/ml in DPPC and 0.2 μg/ml (0.1 μg/ml in parentheses) in DPPC-DPPG (3:1).

[c] Disordered structure.

structure, even in the presence of liposomes. It is interesting that peptide 3_4 also remained in disordered structure in liposomes, in spite of its ability to form α-helix in trifluoroethanol and its weak but appreciable activity against *B. subtilis*.

These results suggest that it is the conformation of the peptide molecules in biological membrane which is important in exhibiting the antimicrobial activity rather than the molecular conformation in aqueous solution.

Phase-transition release

The time-course of the peptide-mediated efflux of carboxyfluorescein from DPPC vesicles is shown in Fig. 4, and the relative abilities of the peptides to efflux carboxyfluorescein from DPPC-DPPG (3 : 1) vesicles are summarized in Table II. At the concentration of 1 μg/ml of the peptides in phosphate buffer (pH 7.4) containing 20 μM DPPC and at the temperature from 0°C to 42°C, the peptides 4_3, $4'_3$, 5_2 and 5_3 released the fluorescent dye completely at the scanning time (5 min). There was no significant release of dye prior to phase-transition (2 min), whereas the release near the phase-transition temperature (40°C) was smooth and rapid. The rate of efflux of the dye mediated by peptide 5_2 was slightly lower than that by peptide 4_3 (Fig. 4). However, peptide 4_3 caused the smooth leakage of dye prior to the phase-transition temperature at the concentration of 10 μg/ml (data not shown). These results suggest that at a low concentration of peptides the leakage of dye from liposomes was not due to the rapid solubilization or to the lysis of the vesicles, but due to the change of the permeability of liposomes. However, the peptides at high concentration might induce the perturbation of liposomes by solubilization or lysis prior to phase-transition temperature. The ability of the peptides to cause dye-leakage from DPPC vesicles containing 25% DPPG is 5-times as high as that from DPPC vesicles alone (Table II), although the leakage profile in DPPC-DPPG vesicles is similar to that in DPPC. These results suggest an electrostatic interaction between peptides and acidic liposomes.

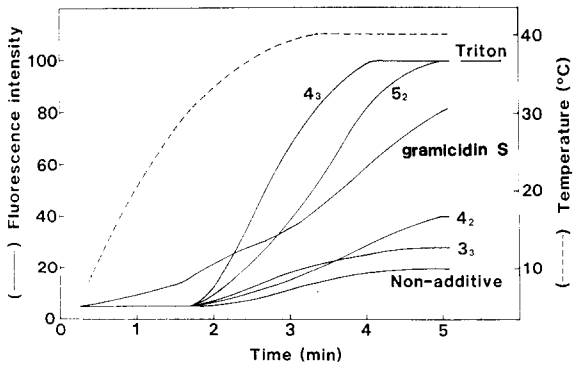

Fig. 4. Profiles of carboxyfluorescein leakage from DPPC vesicles induced by model peptides and gramicidin S. Individual peptides (2 μg) were incubated in 2 ml 0.1 M NaCl/20 mM phosphate buffer (pH 7.4) containing 20 μM DPPC vesicles at temperatures from 0 to 42°C for the indicated scanning time.

The extent of leakage of the fluorescein increased in parallel with antimicrobial activity except for peptide 5_3. The peptides 4_3, 4_4 and $4'_3$, which have high antimicrobial activities, showed a high ability to induce the release of the dye. It should be noted that the mode of dye-leakage of the peptides examined differs from that of gramicidin S which caused weakly the release of dye prior to phase-transition (Fig. 4).

Discussion

We previously reported that the formation of an α-helical structure of the peptides 4_2, 4_3 and 4_4, which have uncharged residues on one side and positively charged residues on the opposite side of the helices, is essential for inducing the perturbation of the membrane structure of mitochondria to facilitate the translocation of precursor protein across the membrane [4]. As demonstrated in the present study, some of the peptides take the α-helical conformation in trifluoroethanol and in the presence of liposomes (Table II and Fig. 3). We illustrate the α-helical net and wheel of the peptides [19] to reveal the relationship between conformation and biological activity (Fig. 5). Arginine residues of 4_3 twist right along the helical axis, and all the Leu and Ala residues reside on the other side of the Arg residues, maintaining an amphiphilic α-helical character. The lower antimicrobial activity of peptide 4_4 than 4_3 might be attributed to the presence of Arg^4, which makes the peptide less amphiphilic because of the

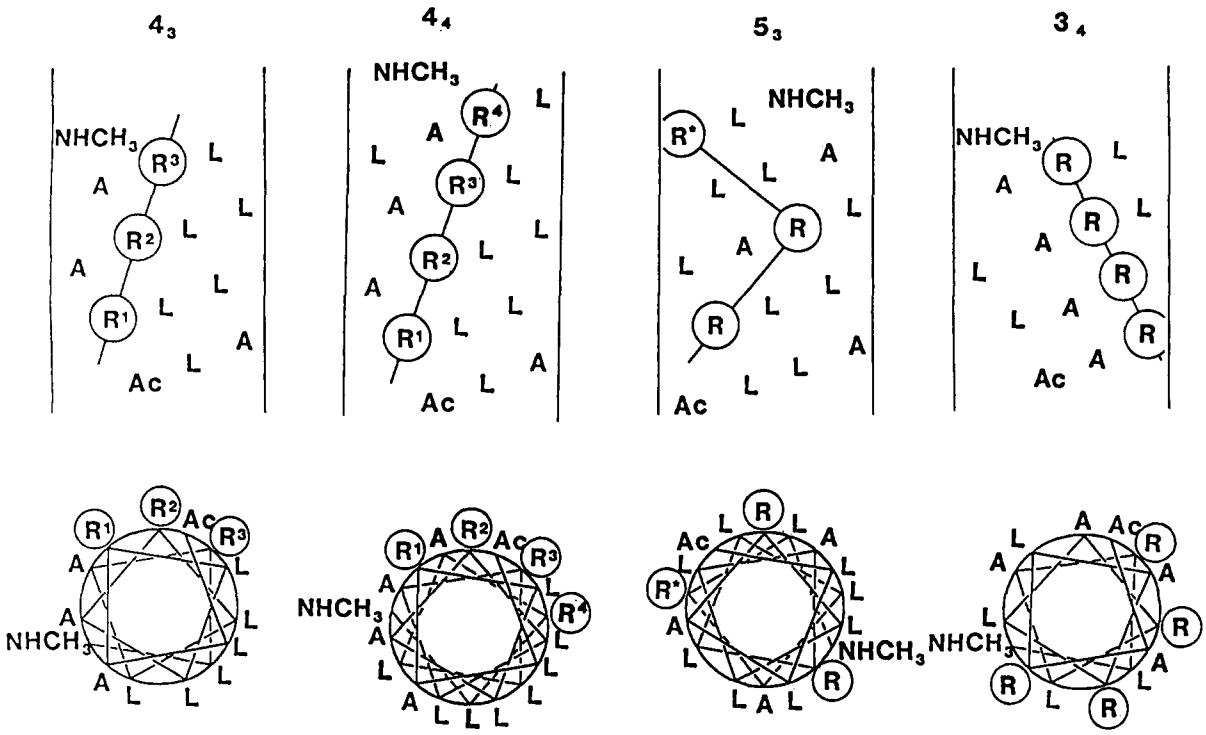

Fig. 5. α-Helical nets and wheels of model peptides 4_3, 4_4, 5_3 and 3_4. One-letter symbols of amino acids are used for concise representation: A, alanine; L, leucine; R., arginine.

location of hydrophilic amino acid (R^4 in 4_4 in Fig. 5) in the hydrophobic region along the helix axis compared with 4_3. Peptide 4_2 can also form α-helix to some extent in the presence of liposomes and consequently shows weak but distinct antimicrobial activity. Disordered structure of inactive peptide 3_3 and slightly active peptide 3_4 in the presence of liposomes could be explained in terms of the decreased amphiphilicity as shown in 3_4 in Fig. 5. Compound 4_3, which has about the same helicity as 3_4 in trifluoroethanol, becomes much helical in the presence of liposomes. We suppose that the lipid bilayer makes 4_3 to be much more energetically stable and amphiphilic by incorporating it into the interface between hydrophobic and hydrophilic phase, and hence the helical content of 4_3 increases. On the other hand, the lipid bilayer keeps 3_4 just on the hydrophilic surface with the charge interaction, resulting in the destabilization of the α-helix. A fairly high antimicrobial activity was found in 5_2, despite the fairly long distance between two Arg residues in this peptide molecule. This might be due to the molecular characteristic that this peptide has a cluster of hydrophobic residues in the one side of the molecule. Contrary to this, 5_3, involving the Arg* residue, showed quite low activity. The presence of the Arg* residue in this peptide seems to be unfavorable to maintain the amphiphilic character of the peptide molecule, as shown in Fig. 5. These results indicate that appropriately cationic amphiphilic peptides which form the α-helix can bind strongly to cell membrane and exhibit antimicrobial activity. Such a view has also been observed for cecropin that is known to form an amphiphilic α-helical structure in the model membrane [6]. The antimicrobial activity of cecropin depends on its ability to form an α-helical structure of the essential N-terminal basic segment [20].

The phase-transition release experiments were carried out to investigate the mode of the lipid-peptide interaction prior to or near at the phase-transition [21]. The experimental data clearly indicated that the peptides like 4_3, 4_4, 5_2 and $4'_3$,

having a high ability to form α-helical structure and high antimicrobial activity, induced complete dye release from DPPC vesicles at the phase-transition temperature of DPPC. This finding implies that the peptides interact preferentially with the liquid crystalline state of liposomes. Epand and Surewicz [22] suggested that such an interaction is ascribable to the intercalation of peptides into the region of the bilayer in the liquid crystalline state. A preferential interaction with the liquid crystal [23] and intercalation into the bilayer [5] have also been observed for melittin, which can form amphiphilic α-helices. Gramicidin S can release weakly the dye from liposomes prior to phase-transition and strongly after phase-transition (Fig. 4). In contrast to the model peptides, gramicidin S may be able to penetrate into the solid state of liposomes due to its much more hydrophobic character by forming a β-structure. However, since peptide 4_3 possessed antimicrobial activity of potency identical to that of gramicidin S, it is plausible that the difference between crystalline and liquid state of liposomes is not critical for exhibition of the biological activity of the peptides.

The peptide 5_3, characterized by the moderate α-helical content, showed a rather low antimicrobial activity and caused the complete dye release. This peptide also interacts strongly with the mitochondrial membrane and has the same potency as that of 4_3 [4]. Such a unique nature of the peptide 5_3 seems to be closely associated with the delicate difference between the nature of lipid bilayers in membrane of bacteria and of mitochondria. It has been known that DPPC vesicles resemble a mitochondrial lipid bilayer, containing a large amount of phosphatidylcholine, rather than a bacterial membrane, which lacks it [24,25].

The model peptides exhibited antimicrobial activity against Gram-positive bacteria but were inactive against Gram-negative bacteria in a manner similar to that of gramicidin S, which has a cationic amphiphilic conformation in the presence of the phospholipid [26]. The amphiphilic structures of the model peptides in liposomes suggest that the mechanism of action of the model peptides is similar to that proposed for gramicidin S [27]. By forming the complex with negatively charged phospholipid on cell membrane, the peptide molecule may penetrate into the membrane to induce the phase separation of phospholipid in the form of frozen segments of the membrane and eventually lead to a disturbance in its barrier properties.

Cecropin and melittin, which can form an amphiphilic α-helical structure in model membrane, show high antimicrobial activity against both Gram-positive and -negative bacteria in contrast to the model peptides and gramicidin S. As to the mode of action of cecropin, Steiner [6] reported that the N-terminal basic amphiphilic helix was anchored to the surface of the membranes and the central lipophilic part of the molecule was penetrated deeply into the lipid bilayer. Okada and Natori [28] reported that sarcotoxin, a family of cecropin, affected signficantly the cell surface of *E. coli* to lose the function of the outer membrane. Assuming that the model peptides take conformations in which the axis of the α-helix face the surface phospholipid bilayer in a parallel manner as seen in the case of an amphiphilic model peptide designed from melittin [29], the model peptides are able to bind to the surface of bilayer of cell membrane in the helical mode. Consequently, the lack of the activity of the peptides against Gram-negative bacteria could be explained by the absence of the hydrophobic part of the molecules which penetrates deeply in lipid bilayer to perturb the outer membrne of the bacteria.

In conclusion, the results presented herein indicate that amphiphilicity, i.e., appropriate positioning of cationic and hydrophobic groups constructed in the stable α-helical structure in the peptides, is a significant factor requisite for exhibiting antimicrobial activity against Gram-positive bacteria. Based on the fact that the peptides which can interact strongly with DPPC vesicles as shown by the results of CD and phase-transition experiments exhibited the high antimicrobial activity, it can be postulated that the disturbance of the membrane potential is caused as a consequence of shallow penetration of the hydrophobic domains into the bacterial membrane [8] and the interaction of the basic amino-acid residues located at the hydrophilic parts with acidic moiety of phospholipids in the membrane of Gram-positive bacteria.

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