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Antimicrobial specificity and hemolytic activity of cyclized basic amphiphilic β-structural model peptides and their interactions with phospholipid bilayers

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We synthesized a series of cyclic antiparallel β-sheet model peptides with various ring sizes, which were designed on the basis of a cyclic β-structural antibiotic, gramicidin S (GS); cyclo(Val-Orn-Leu-D-Phe-Pro)$_2$, and investigated in terms of their antimicrobial activity and specificity against Gram-positive and Gram-negative bacteria and lytic activity for human erythrocytes. In our planning, in order to compare the peptides with GS, D-Phe-Pro sequence forming β-turn in GS molecule remained unaltered and repeating sequences of alternately hydrophobic (Leu)-hydrophilic (Orn) residue were introduced into the β-structural parts. CD study in acidic liposomes as well as leakage study of carboxyfluorescein encapsulated in phospholipid vesicles indicated that the peptides strongly interacted with lipid bilayers by taking an amphiphilic β-structure. Antimicrobial study showed that although GS is active only against Gram-positive bacteria, the antimicrobial spectra of the model peptides transformed gradually to be active against Gram-negative ones and finally only against Gram-negative bacteria whose repeating sequences increased. It should be noted that the designed cyclic model peptides show antibacterial activity but accompany no hemolysis. This indicates that an appropriate hydrophobicity together with a proper orientation of hydrophilic (cationic) and hydrophobic groups in cyclic β-structural molecules can hold antimicrobial activity against both types of bacteria without damaging eukaryotic cells.

Introduction

Amphiphilic β-structural peptides isolated recently from natural sources are known to show a cytolytic effect in vitro against bacteria and parasites, presumably contributing to the defense mechanisms of the host animals against invading microorganisms. The common feature of the amphiphilic β-structural peptides is the presence of a considerable number of basic residues with a relatively high number of hydrophobic amino acid residues in their cyclic molecules and broad-spectrum antimicrobial activity.

Gramicidin S (GS) from certain strains of bacillus brevis has strong antimicrobial activity against Gram-positive, but none against Gram-negative bacteria, in addition to strong lytic activity against erythrocytes [1,2]. Contrary to this, other peptides such as defensin [3] and bactenesin [4] from neutrophils of vertebrates and tachyplesin [5] from the hemocyte debris of anthropoda which constitute the animal defense systems have broad-activity against microorganisms including Gram-positive and negative bacteria, but no lytic activity against erythrocytes. Relating to this, we have previously reported that a macro ring analog of GS having 14-membered residues with increasing basic residues in the molecule has antimicrobial activity against both Gram-positive and negative bacteria [6]. However, the reason why β-structural peptides show the lytic specificity for both types of bacteria and animal cells is not yet well interpreted.

To clarify the reason, GS seems to be basically a good model compound because it is an amphiphilic β-structural peptide having hydrophobic side chains of Leu and Val on the one side and hydrophilic side chains of ornithine on the other side. GS interacts with

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Abbreviations according to IUPAC-IUB Commission (1984) Eur. J. Biochem, 138, 9-37, are used throughout. Other abbreviations: DPPC, dipalmitoyl-DL-α-phosphatidylcholine; DPPG, dipalmitoyl-DL-α-phosphatidylglycerol; egg PC, egg-yolk phosphatidylcholine; egg PG, egg-yolk phosphatidylglycerol; GS, gramicidin S; Tris, tris(hydroxymethyl)aminomethane; Z, benzyloxycarbonyl; Boc, *t*-butyloxycarbonyl. All amino acid symbols denote L-configuration.

cyclo(Val$^{1,1'}$-Orn$^{2,2'}$-Leu$^{3,3'}$-D-Phe$^{4,4'}$-Pro$^{5,5'}$)$_2$	GS
cyclo(Orn-Leu-Orn-D-Phe-Pro)$_2$	DLL-10
cyclo(Orn-Leu-Orn-Phe-Pro)$_2$	LLL-10
cyclo(Leu-Orn-Leu-Orn-D-Phe-Pro-Orn-Leu-Orn-Leu-D-Phe-Pro)	DLL-12
cyclo(Leu-Orn-Leu-Orn-Leu-D-Phe-Pro)$_2$	DLL-14
cyclo(Val-Orn-Val-Orn-Val-D-Phe-Pro)$_2$	DLV-14
cyclo(Leu-Orn-Leu-Orn-Leu-Orn-Leu-D-Phe-Pro)$_2$	DLL-18

Fig. 1. Peptides designed and synthesized.

various membranes, such as bacteria, mitochondria, erythrocytes and model membranes. For the antimicrobial activity or for binding to membrane, the positive charges have been considered to be essential [2]. However, the role of cyclic structure and basic residues in showing the lytic specificity has not been clarified yet. Therefore, we synthesized the following cyclic deca-, dodeca-, tetradeca-, and octadecapeptides as shown in Fig. 1. For these peptides, the relation between structure and activity was examined by measuring CD spectra, membrane perturbation ability and antimicrobial and hemolytic activity.

Materials and Methods

Materials

GS, Dipalmitoyl-DL-phosphatidylcholine (DPPC), Dipalmitoyl-DL-phosphatidylglycerol (DPPG), egg-yolk phosphatidylcholine and egg-yolk phosphatidylglycerol were purchased from Sigma. Carboxyfluorescein purchased from Eastman Kodak was purified by recrystallization from a mixed solution of methanol and water. All other reagents were of analytical grade. The peptide concentration in solution was determined by amino acid analysis. Phospholipid concentration was determined by an assay using the phospholipids-test reagent purchased from Wako Pure Chemical Industries (Osaka, Japan), and it was expressed in terms of phosphorus concentration.

Synthesis of peptides

The peptides, DLL-10, LLL-10, DLL-12, DLL-14, and DLV-14, were synthesized by the same method as described in the previous papers [6–8].

Cyclic octadecapeptide, DLL-18, newly designed in the present paper, was synthesized by solution method. The synthetic process of DLL-18 is outlined in Fig. 2. Boc-tetrapeptide ester (**1**) was prepared by the condensation of the azide derived from Boc-Leu-Orn(Z)-$NHNH_2$ [7] and H-Leu-Orn(Z)-OEt · HCl. Boc-tetrapeptide hydrazide (**2**) prepared from **1** by treatment with hydrazine was converted to the azide, which was condensed with H-Leu-Orn(Z)-Leu-D-Phe-Pro-OEt · HCl [7] to give Boc-nonapeptide ester (**3**). The Boc-nonapeptide hydrazide (**4**) prepared from **3** and nonapeptide ester hydrochloride (**5**) obtained from **3** by treatment with HCl in formic acid was condensed to give Boc-octadecapeptide ester (**6**) by the azide method. The compound was followed to hydrazinolysis, and deprotection of Boc-group by HCl in formic acid, and then the azide obtained by treatment of isopentyl nitrite was cyclized in a large amount of pyridine (approx. $3 \cdot 10^{-3}$ M of peptide concentration). The crude product obtained was passed through the columns of Dowex 50, Dowex 1, and Sephadex LH-20 to afford Z-substituted cyclic octadecapeptide (**7**). The compound was confirmed to be monomeric by measuring molecular weight in dimethylformamide with a Corona Osmometer Type 117; the theoretical and found molecular weights were 2884 and 3072, respectively. The desired cyclic octadecapeptide (DLL-18) · 6HCl was obtained as needle crystals after hydrogenation of **7** in the presence of palladium black in methanol containing hydrogen chloride; m.p. 245–250°C (decomp.), $[\alpha]_D^{25}$ −40.0° (*c*, 0.07, MeOH). The homogeneity of DLL-18 was ascertained by means of thin-layer chromatography, paper electrophoresis, and elemental analysis (as DLL-18 · 6HCl · $9H_2O$). Amino acid analysis was carried out using a JASCO HPLC amino acid analysis system equipped with FP-210 spectrofluorometer as a detector after hydrolysis of the peptide in 6 M HCl at 110°C for 24 h. The amino acid ratio was Leu (4.10), Orn (3.90), Phe (1.00), and Pro (1.09).

Preparation of phospholipid liposomes

Phospholipid (20 mg, about 27 μmol) was dissolved in chloroform (1 ml) and dried by breathing of nitrogen in a conical glass tube. The dried lipid was hydrated in 2 ml of 20 mM Tris-HCl buffer (pH 7.4) with repeated

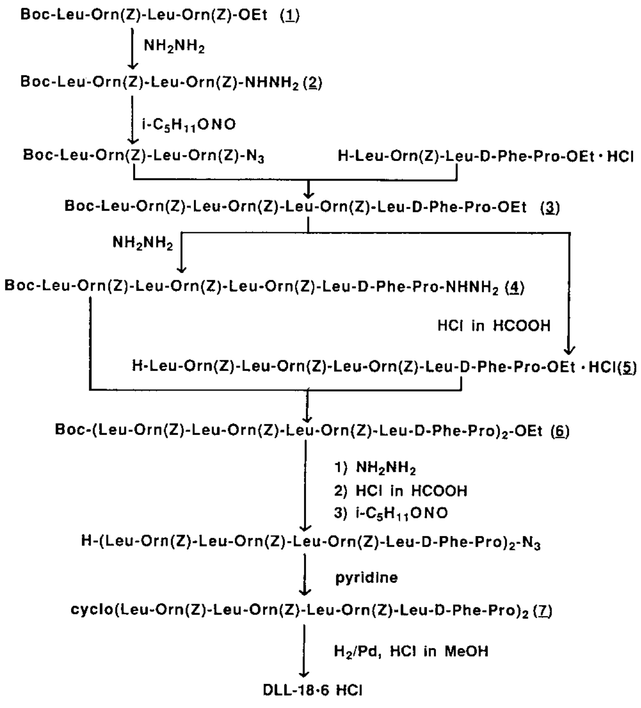

Fig. 2. Synthetic route of DLL-18.

Vortexed-mixing at 50°C for DPPC and DPPG or at 25°C for natural phospholipids for 30 min. The suspension was sonicated at 50°C or 25°C for synthetic or natural lipid, respectively, for 60 min using a Branson Sonifier Model 185 and diluted to 25 ml with the same buffer (lipid concentration, about 1.0 mM). The mixture of uni- and multilamellar vesicles obtained was used for the CD measurement. The unilamellar vesicles trapping carboxyfluorescein were prepared by the same method as described above except that the dried lipid (20 mg, 27 μmol) was hydrated in 2 ml of 0.1 M NaCl/20 mM phosphate buffer (pH 7.4) containing 100 mM carboxyfluorescein. After sonication the mixture of uni- and multilamellar vesicles trapping carboxyfluorescein was subjected to gel filtration through a Sephadex 4B column (1 × 20 cm) in 0.1 M NaCl/20 mM phosphate buffer (pH 7.4). 1-ml fractions were collected and the solution of fraction numbers 13–15 that were just before the non-encapsulated dye-elution was collected to utilize for carboxyfluorescein release measurements. The lipid concentration of the fractions 13–15 was about 3.0 mM.

CD measurement

CD spectra were recorded on a JASCO J-600 spectropolarimeter using a quartz cell of 0.1 cm pathlength. Spectra in 20 mM Tris-HCl buffer (pH 7.4) or in methanol were measured at a peptide concentration of 100 μM. Spectra in the presence of phospholipid liposomes were measured at a peptide concentration of 10 μM in 20 mM Tris-HCl buffer (pH 7.4) containing 0.9 mM vesicles. When the solution became cloudy after the addition of peptides to liposome solution, it was sonicated again to become clear with a bath type sonicator. All the measurements were carried out at 25°C. The CD data were expressed in terms of the molar ellipticity.

Leakage of carboxyfluorescein from phospholipid vesicles

A liposome solution (50 μl) of fractions 13–15 obtained by passing through Sepharose 4B was added to a 2 ml of phosphate buffer in the cuvette to give a final concentration of 75 μM lipid. To the mixture was added a 0–70 μl of an appropriate concentration of the peptide in phosphate buffer. Fluorescence spectra were recorded on a JASCO FP-550A spectrofluorophotometer. The cuvette was placed in the heated cuvette holder of the fluorometer at 25°C and the fluorescence intensity was continuously recorded. The intensity was monitored by fluorescence at 515 nm with exciting at 470 nm. The data were collected at 3 min after the addition of the peptides in the vesicle solution. For determination of the fluorescence intensity derived from 100% dye-release, 10 μl of Triton X-100 solution (20% in phosphate buffer) was added to dissolve the vesicles. The percentage of 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 of the fluorescence without the peptides and with Triton X-100 treatment, respectively.

Hemolytic assay

The buffy coat was removed by centrifugation of freshly collected human blood, and the erythrocytes obtained were washed three times with isotonic saline and stored at 4°C. They were incubated with peptides at 37°C in 10 mM Tris-HCl containing 150 mM NaCl buffered saline, and then centrifuged. The absorbance in the supernatant was measured at 540 nm. Here, the absorbance of the supernatant obtained by treatment of erythrocytes with 1% Triton X-100 was taken as 100%.

Antimicrobial assay

The minimum inhibitory concentration (MIC) was determined by the standard agar dilution method using trypticase soy agar as described by Okonogi et al. [9].

Results

Design and synthesis of peptides

Fig. 1 shows primary structures of peptides used in the present study. A part of possible secondary structures of the peptides is shown in Fig. 3. As shown in Fig. 3a, the peptide backbone of GS holds a rigid antiparallel β-structure stabilized by four intramolecular hydrogen bonds two of which form type II′ β-turn. We planned to synthesize such model peptides holding a GS-like antiparallel β-pleated sheet structure. Therefore, the D-Phe-Pro sequence capable of taking type II′ β-turn conformation with high potency was remained unaltered. As the β-structural parts in each molecule, the repeated sequences of alternating hydrophobic-hydrophilic amino acid residues were selected. Such repeated sequences are known to have an amphiphilic structure when they adopt β-structure [20]. The amino acids, Leu and Orn, were applied as hydrophobic and hydrophilic residues, respectively.

DLL-10 is a cyclic decapeptide similar to GS, but its four hydrophobic residues (Leu and Val) and two hydrophilic residues in antiparallel β-pleated sheet are replaced by Orn and Leu residues, respectively. This peptide will be less hydrophobic than GS when it takes a β-structure similar to GS (Fig. 3a). LLL-10 is similar to DLL-10 (Fig. 3b), but the L-Phe-Pro sequence in molecule differs from that.

DLL-12 is a cyclic dodecapeptide in which valine residues of the GS is replaced to leucine residues and possesses two more ornithine residues than GS. If DLL-12 has such a type II′ β-turn as GS structure, it would take an amphiphilic structure that is segregated

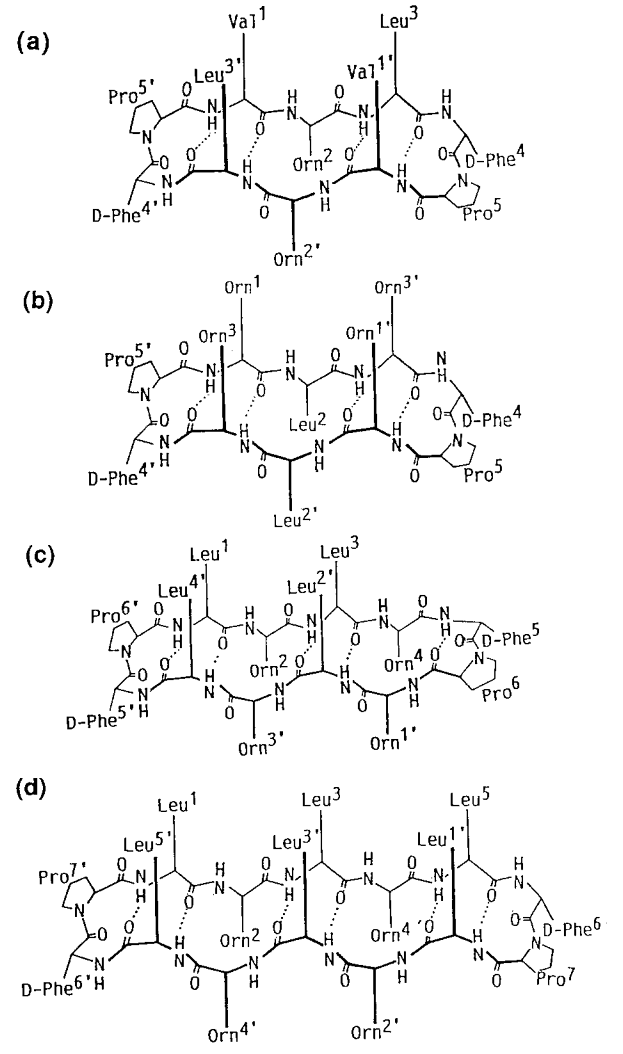

Fig. 3. Secondary structures of GS (a), DLL-10 (b), DLL-12 (c), and DLL-14 (d).

with the hydrophobic site on one side of the peptide-ring and with the hydrophilic site on the other side (Fig. 3c).

DLL-14 and DLL-18 are cyclic tetra- and octadecapeptides containing four and six ornithine residues with two and three Leu-Orn repeating sequences in antiparallel β-sheet structures, respectively (Fig. 3d shows DLL-14).

To investigate the effect of the valine and leucine residues on antibacterial activity and conformation, we also designed a cyclic tetradecapeptide, DLV-14, containing six valine residues instead of six leucine residues in DLL-14.

Antimicrobial activity

The minimum inhibitory concentration of the peptides is shown in Table I. DLL-14 has considerable potencies to inhibit the growth of both Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* and Gram-negative bacteria such as *Escherichia coli* and *Shigella flexneri*, as reported previously [7]. Substitution of Leu to Val in DLL-14 decreases drastically its antimicrobial activity as observed in DLV-14. DLL-12 having same basic residues and smaller ring size in comparison with DLL-14 shows a similar inhibitory potency as DLL-14. However, DLL-10 that has same basic residues and much smaller ring size as compared with DLL-14 shows a very weak antimicrobial activity against both bacteria. Surprisingly, LLL-10 that has same ring size and basic residues as compared with DLL-10 shows almost the same antimicrobial activity as DLL-14. It is worth noting that DLL-18 shows a considerable activity against Gram-negative bacteria and no activity against Gram-positive ones, i.e., the antimicrobial spectrum of which reverses that of GS.

CD study

In order to investigate the conformations of analogs, we measured CD spectra in methanol, in the presence and absence of neutral or acidic phospholipid liposomes in Tris-HCl buffer (pH 7.4) at 25°C as shown in Fig. 4. GS has a double minimum at 206 nm and at 217 nm in all media (Fig. 4a). The trough becomes deeper by addition of liposomes but is not so changeable, on the whole, in all media, suggesting that GS keep a rigid conformation on binding to lipid bilayers. Our detailed experiment using cyclo(-D-Phe-Pro-ω-aminocaproyl)

TABLE I

Antibacterial activity of model compounds and GS

Medium: trypticase soy agar. Inoculum: 5 μl of a bacterial suspension containing 10^7 CFU ml^{-1}

Organism	Minimum inhibitory concentration (μg ml^{-1})						
	DLL-10	LLL-10	DLL-12	DLL-14	DLV-14	DLL-18	GS
Staphylococcus aureus FDA 209P	> 100.0	50.0	25.0	12.5	> 100.0	> 100.0	6.25
Bacillus subtilis PCI 219	50.0	12.5	6.25	6.25	50.0	> 100.0	3.13
Escherichia coli NIHJ JC-2	> 100.0	50.0	12.5	12.5	> 100.0	12.5	> 100.0
Salmonella typhosa Boxhill 58	> 100.0	25.0	> 100.0	25.0	> 100.0	> 100.0	> 100.0
Shigella flexneri EW-10	50.0	6.25	12.5	6.25	100.0	25.0	> 100.0
Shigella sonnei EW-33	100.0	25.0	12.5	12.5	> 100.0	25.0	100.0
Klebsiella pneumoniae DT	100.0	25.0	12.5	12.5	> 100.0	6.25	> 100.0

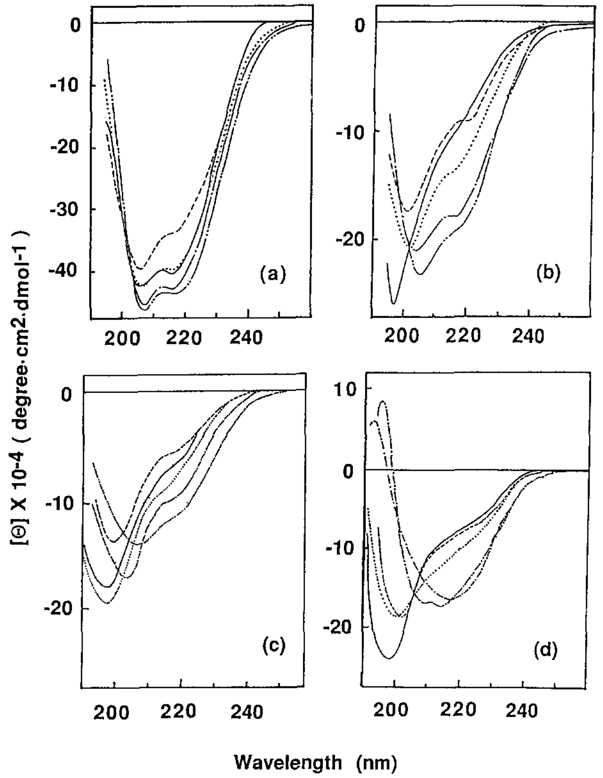

Fig. 4. CD spectra of GS(a), DLL-14 (b), DLV-14(c), and DLL-18(d). Spectra in methanol and Tris-HCl buffer were measured at a peptide concentration of 100 μM at 25°C. Multilamellar vesicles were prepared by sonication. Peptides were dissolved at the concentration of 10 μM in Tris-HCl buffer containing 0.9 mM liposomes. Methanol (– – – –); Tris-HCl buffer (———); and DPPC (······); DPPC/DPPG (3:1) (–·–·–); DPPG (–··–··–).

has shown that the CD spectrum of the type II′ β-turn formed by D-Phe-Pro has a strong negative minimum band at 206 nm with a shoulder at near 213 nm [10]. Since the CD spectrum of β-structural poly(L-lysine) has a negative minimum band at 217 nm [11], it is clear that the double minimum band of GS is attributable to a combined trough of the β-sheet structure and the β-turn.

As reported in the previous paper [7], the CD spectrum of DLL-14 in methanol is similar to that of GS and the trough is shallower than that of GS (Fig. 4b). The CD spectrum in water or in Tris-HCl buffer (pH 7.4) shows a strong negative band at 196 nm which is characteristic of a random structure. Upon the addition of phospholipid liposomes, the spectrum considerably changes and becomes very similar to that of GS, although the ellipticity is smaller than that of GS. Particularly, in the case of acidic liposome (DPPG), the shape is quite similar to that of GS, indicating that DLL-14 forms an antiparallel β-sheet structure with a β-turn similar to GS in the presence of the liposomes. Because the DLL-14 is considered to have a larger flexibility as compared to GS molecule, it cannot retain an antiparallel β-structure in water. The amphiphilic β-structure is first formed by the addition of liposomes. The ellipticities of the double minima in DLL-14 in the presence of acidic phospholipid liposomes (DPPG) are much larger than those in neutral liposomes such as DPPC. The result indicates that the charge interaction of cationic side chains of DLL-14 and the head group of acidic phospholipids also promotes the formation of β-structural conformation. DLL-12 shows a similar behavior in CD spectra in all media to DLL-14 (data not shown).

The spectra of DLL-10 having the same ring size as GS are attributable to a mixture of a random coil and a β-structure with β-turns in water and GS-like conformation in acidic liposomes, but those of LLL-10 take random-like curves in all media (data not shown).

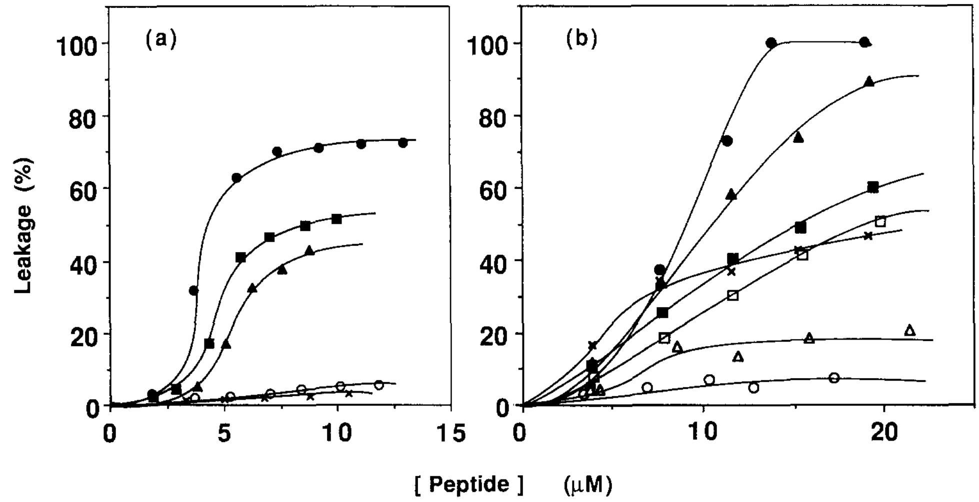

Fig. 5. Leakage profiles of carboxyfluorescein from DPPC/DPPG (3:1)(a) and egg PC/egg PG (3:1) (b) vesicles by GS and the model peptides. Individual peptides were incubated at 25°C as described in Materials and Methods. GS (●); DLL-10 (○); LLL-10 (×); DLL-12 (■); DLL-14 (□); DLV-14 (▲); DLL-18 (△).

On the other hand, as shown in Fig. 4c, the cyclic tetradecapeptide DLV-14 takes a spectral pattern responsible for a random coil in methanol and buffer solution. When acidic phospholipid (DPPG) is added to Tris-HCl buffer (pH 7.4), the spectra transform to GS-like pattern. However, the ellipticities of the double minimum peaks are much smaller than those of DLL-14. This result suggests that DLV-14 takes a less stable structure than DLL-14, probably because of more steric hindrance and/or less hydrophobicity of Val residue as compared with Leu.

The cyclic octadecapeptide (DLL-18) has a different spectral pattern compared to that of GS (Fig. 4d). In methanol and in the presence of neutral liposomes, DLL-18 has a strong negative band at near 200 nm with a shoulder at about 215 nm, suggesting that it mainly takes a random structure. On the other hand, it has double minima at 210 nm and 216 nm in a mixture of DPPC/DPPG (3 : 1), and interestingly a broad negative band at 220 nm in DPPG is seen. These results reveal that the CD curve in DPPG liposomes is mainly attributable to β-pleated sheet moiety in the macro ring molecule.

Nearly similar tendency in CD spectra is observed when a mixture of natural phospholipids of egg PC and egg PG was used in stead of synthetic phospholipids (data not shown). This suggests that the peptides interact equally with gel or fluid phase of the bilayers.

Leakage of liposome content

It is known that the interaction of amphiphilic peptide with lipid bilayers can alter the structure and change the permeability of lipid bilayers. The leakage of encapsulated carboxyfluorescein from the vesicles can be easily used for examining such peptide–lipid interactions [12].

The extent of carboxyfluorescein release from small unilamellar DPPC/DPPG (3 : 1) vesicles is shown as a function of the peptide concentration in Fig. 5a. Among the peptides, GS can leak the dye with the highest ability from DPPC/DPPG (3 : 1) vesicles. Its leakage ability increases rapidly at low concentration (below 7 μM) but it reaches plateau with about 70% dye-release at about 10 μM, and further release is not observed in the range of the concentration examined. The intensity of efflux of the dye mediated by DLL-12 or DLL-14 is lower than that mediated by GS and decreases in the following order; GS, DLL-12, DLL-14, DLL-10, and LLL-10. Moreover, the order of the intensity decrease is parallel to that of antimicrobial activity against Gram-positive bacteria. Note that the cyclic decapeptide LLL-10, which has relatively high antimicrobial activity, accompanies no leakage, and that, on the other hand, DLL-10 shows neither antimicrobial activity nor leakage (see Fig. 5a).

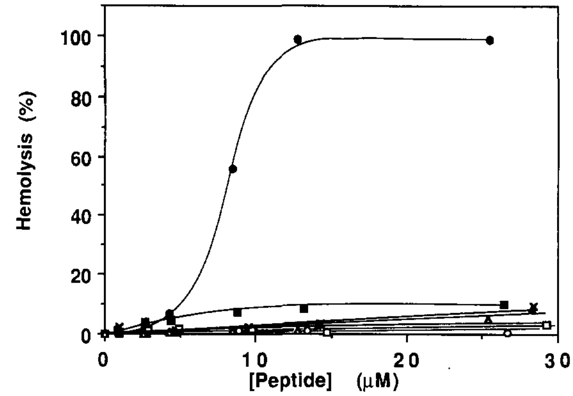

Fig. 6. Erythrocyte lysis by GS and the model peptides. An erythrocyte suspension was exposed to varying concentration of GS and the model peptides. The amount of hemoglobin released was determined spectrophotometrically by measuring the absorbance of 540 nm. Complete lysis (100%) was obtained by the addition of Triton X-100 to the erythrocyte suspension. GS (●); DLL-10 (○); LLL-10 (×); DLL-12 (■); DLL-14 (□); DLV-14 (▲); DLL-18 (△).

Similar results were obtained in egg PC/egg PG (3 : 1) liposomes, although all the peptides released the dye more strongly as compared to DPPC/DPPG (3 : 1) (Fig. 5b). It is of interest that DLV-14 having weak antimicrobial activity shows a relatively high leakage ability and DLL-18 has a much weaker ability as compared to DLL-14. In general, the peptides having relatively high activity against Gram-negative bacteria are likely to release the dye weakly.

Interaction of the peptides with red blood cells

Fig. 6 shows the hemolysis of erythrocytes as a function of the concentration of the peptides. GS lyses the erythrocyte very little up to 4 μM. Beyond 4 μM, considerable lysis is observed and 100% lysis is observed at 12 μM. On the contrary, all the model peptides scarcely lyse the erythrocytes at the concentration employed (30 μM).

Discussion

Amphiphilic structure, which is formed from sterically allowed secondary structures, namely, α-helix, β-pleated sheet structure, 3_{10}-helix or π-helix, has been found in biologically active peptide molecules [13]. In particular, the basic amphiphilic α-helical structure is considered to be one of the most important structural units for antimicrobial activity as found in naturally occurring peptides as melittin [14], cecropin [15], magainin [16] and dermaceptine [17] and in model peptides as Ac-(Leu-Ala-Arg-Leu)$_2$ or $_3$-NHCH$_3$ (4_2) or (4_3) [18]. On the contrary, characteristic feature of antimicrobial β-structural peptides isolated from natural source such as defensin [3], bactenesin [4] tachyplesin [5] possesses a cyclic structure restricted by amide bond or S-S bridge. We have previously reported that linear basic amphiphilic β-structural peptides, Ac-(Val-Lys)$_4$-NHCH$_3$, and Ac-(Ser-Val-Lys-Val)$_3$-NHCH$_3$ exhibit no antimicrobial activity [19]. These suggest that amphiphilic α-helical peptides show strong antimicrobial activity with linear structure, while those of β-structure seem to need cyclic antiparallel β-structure. The results presented here by using various β-forming cyclic peptides provide an insight into clarifying the structure–activity relationship and the specificity for lysis of bacterial membranes and mammalian cells.

As has been described already, the cyclic model peptides consist of alternative hydrophobic and hydrophilic amino acid residues and also have β-turn formed by D-Phe-Pro sequence. These model peptides could form amphiphilic β-structure in amphiphilic surroundings such as lipid bilayers. CD spectroscopic data indicated that (i) cyclic decapeptide DLL-10 and cyclic dodecapeptide DLL-12 took a β-structural conformation in aqueous solution and in the presence of acidic and neutral phospholipids, and (ii) macro-ring peptides DLL-14 and DLL-18 existed taking the following conformations: in buffer solution, mainly unordered structure; in the presence of neutral liposomes, a mixture of unordered and β-structural conformation; and in the presence of acidic liposomes, a β-structural one. It is certain that the chain length of antiparallel β-structural part connected with β-turns is one of the important factors on maintaining its structure. It is probable that in shorter chain length of the β-structural part, intramolecular hydrogen bond(s) between antiparallel β-chains will be stabilized by the influence of the stable β-turn, but those in longer chain are less stable. Therefore, while macro-ring compounds are not at all or less capable of forming β-structure in aqueous solution, hydrophobic environments such as liposomes assist the formation of β-structure, so they can adopt the amphiphilic β-structure. The charge interaction of basic peptides with acidic phospholipid-head groups is also important to induce β-structure. These results are consistent with the previous view that the oligopeptides consisting of alternating hydrophobic (Val) and hydrophilic amino acid (Lys) are able to form amphiphilic β-structure in acidic phospholipid bilayers, but in aqueous solution and in neutral phospholipid bilayers, they form hardly such structure. It should be noted that although Val possesses the highest β-structure forming potential and DLV-14 consists of six Val residues, contrary to expectation, DLV-14 less prominently forms the β-structure than DLL-14 [21] (see Fig. 4c). This discrepancy suggests that on amphiphilic surface such as liposomes, the peptide consisting of Leu residues whose hydrophobicity is greater than Val, adopts amphiphilic β-structure by interacting more easily with lipid core of phospholipid bilayers.

Turning to the data shown in Table I, antimicrobial assay of the model compounds exhibited an interesting result. That is, although GS is active only against Gram positive bacteria, the activities of the model compounds are found to transform gradually toward against Gram-negative ones with increasing in the basic residue number. As to Gram-positive bacteria, the order of antimicrobial activity is GS > DLL-14 > DLL-12 > LLL-10 > DLL-10, DLV-14 > DLL-18. This is almost parallel with the ability of the release of encapsulated carboxyfluorescein from acidic liposomes (except for LLL-10), indicating that the antimicrobial activity of the β-structural peptides against Gram-positive bacteria may be represented by the affinity of binding of the peptide to lipid bilayers. Relating to this, physicochemical studies have indicated that GS is inserted into the hydrophobic core of DPPC liposome and immobilize boundary lipids [22]. Since the extent of dye release is considered to be proportional to the insertion of the peptides into lipid bilayers, the decrease of antimicrobial activity against Gram-positive bacteria in macro-ring peptides may correspond to that of their hydrophobicities. Exceptionally, LLL-10 which does not take β-structure even in the presence of acidic liposome has considerable activity against Gram-positive bacteria but causes no leakage. This peptide may interact with the biomembranes by a certain different mechanism.

The increase of two basic residues (Orn) compared to GS leads to enhancement of the anti-microbial activity against Gram-negative bacteria. However, this trend is not the case for DLL-10 and DLV-14. It is well-known that the outer membrane acting as a protector of Gram-negative cells against most hydrophobic antibiotics is relatively ineffective at cationic antibiotics [23]. Since an increase in basic residue number leads to a decrease in hydrophobicity, the results described above are reasonable. It is interesting that DLL-18 having more two basic residues than DLL-14 possesses no activity against Gram-positive bacteria but considerable activity against Gram-negative ones. This is absolutely opposite to GS in antimicrobial spectra.

We previously reported that the sequential oligomers of (Leu-Orn)$_n$ ($n = 2$–12) [24] and Ac-(Val-Lys)$_n$-NHCH$_3$ ($n = 2$–4) taking amphiphilic structure in acidic liposomes [19] have shown no antimicrobial activity neither against Gram-positive nor against negative bacteria. The present study indicates that a certain rigid structure like cyclic structure in addition to amphiphilic β-structure structure is proposed to show antimicrobial activity. In this connection, there have been a few reports that a random copolymer of Leu and Orn with average residue of twenty-three [25] and poly(L-lysine) with average residue of fifty inhibit the growth of *E. coli* [26]. Therefore, there remains a possibility that the simple increase of basic residue in molecule may be responsible for antimicrobial activity against Gram-negative bacteria. This is interpreted from the fact that LLL-10 taking no β-structure shows antimicrobial activity against both bacteria.

As was described already, DLL-14 showed substantial activity against both Gram-positive and Gram-negative bacteria, in contrast, DLV-14 exhibited weak activity against only *Bacillus subtilis* in Gram-positive bacteria but no activity against Gram-negative ones. This result indicates that most of the leucine residues in DLL-14 cannot be replaced by valine residues without serious influence for antibacterial activity. As mentioned above, the hydrophobic part consisting of leucyl side chains in DLL-14 is more hydrophobic on taking an amphiphilic conformation than that of valyl side chain in DLV-14. Relating to this, it has been reported that [Leu$^{1,1'}$]-GS [27] exhibits considerable antibacterial activity against Gram-positive bacteria while [Val$^{3,3'}$]-GS [28] or [Ala$^{1,1' \text{ and } 3,3'}$]-GS [29] exhibits weak or no activity against Gram-positive bacteria, respectively.

The model peptides have no lytic activity against human erythrocyte, while GS lyses strongly. The naturally occurring β-structural peptides which serve as a defense system need to have broad-spectrum and strong activity against both bacteria and fungi. Therefore, β-structural peptides are desired to cause no lysis of the mammalian or insect cells. Related to this, incorporation of thymidine into B-cell by bacterial lipopolysaccaride, accelerating such incorporation [30], was not inhibited by DLL-14 (unpublished data). This phenomenon was also observed for GS but simultaneously cell lysis did take place, indicating that DLL-14 does not lyse B-cell which constructs the defense system. Such specificity of the peptides is probably determined by the hydrophobicity, conformation, and the chemical nature of hydrophobic and cationic sites as proposed by Kini and Evans [31]. The results presented here indicate that the appropriate hydrophobicity as well as the proper orientation of hydrophilic (cationic) and hydrophobic groups in cyclic molecule is requisite not only for exhibiting antibacterial activity against several bacteria but also for causing no damage on host cells.

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