Journal of Peptide Science
J. Peptide Sci. **5**: 298–305 (1999)

Effect of Amino Acid Substitution in Amphiphilic α-Helical Peptides on Peptide–Phospholipid Membrane Interaction

TAKURO NIIDOME, SHINGO ANZAI, JUNKO SONODA, YOKO TOKUNAGA, MASAAKI NAKAHARA, TOMOMITSU HATAKEYAMA and HARUHIKO AOYAGI*

Department of Applied Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki, Japan

Received 15 October 1998
Accepted 18 December 1998

Abstract: It was previously found that a cationic amphiphilic peptide, Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$ (**4$_3$**), caused the destabilization of a phospholipid membrane and showed strong antibacterial activity [Lee *et al. Biochim. Biophys. Acta* 1986; **862**: 211–219]. In order to investigate the effect of changing α-helix propensity, hydrophobicity and basicity in **4$_3$** on the peptide conformation and activity, the **4$_3$** analogs, [Gly (or Val)^6]**4$_3$**, [Gly (or Val)^{2,6}]**4$_3$**, [Gly (or Val)^{2,6,10}]**4$_3$**, [Gln^3]**4$_3$**, [Gln^{3,7}]**4$_3$** and [Gln^{3,7,11}]**4$_3$** were synthesized. Except for [Val^{2,6}]**4$_3$** and [Val^{2,6,10}]**4$_3$**, which mainly formed a β-structure, other peptides formed an α-helix and showed moderate membrane-perturbing activity toward neutral and acidic lipid vesicles. All the peptides other than [Val^{2,6,10}]**4$_3$** and [Gln^{3,7,10}]**4$_3$** had the antibacterial activity comparable with that of **4$_3$**. The relationship between the membrane-perturbing activity and the antibacterial activity was not always parallel. Conclusively, the Ala→Val substitution in **4$_3$** causes the change of peptide conformation and the presence of a cationic amino acid residue is necessary for the antibacterial activity. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: amphiphilic peptide; circular dichroism; membrane perturbation; hemolytic activity; antibacterial activity

INTRODUCTION

Many naturally occurring and artificial amphiphilic α-helical peptides have been found to show a variety of biological activities, e.g. membrane perturbation [1–4], membrane fusion [5–7], antimicrobial activity [8–10], and ion channel formation [11–14]. In these cases, the properties and behaviors of peptides, which are governed by hydrophobicity, helicity and amphiphilicity, are different from each other when in an aqueous environment and in a hydrophobic one. This suggests that the character of amino acids in peptides is influenced by their surroundings. With this in mind, Deber *et al.* have systematically investigated the effect of successive substitution of guest amino acids in a host peptide on the peptide conformation and property [15,16]. They found that Ile and Val are not helix breakers but rather helix promoters in a membrane-mimic environment; in other words, the helical propensity of these amino acids is enhanced in the helix-favoring environment of a membrane bilayer [15]. Scheraga *et al.* also reported that the disrupting effect of a Pro residue in an α-helix depends on its location in the peptide [17].

We previously presented that an amphiphilic α-helical peptide, Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$ (**4$_3$**),

Abbreviations: CD, circular dichroism; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; FAB-MS, fast atom bombardment mass spectrometry; HBTU, *O*-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; PBS, phosphate-buffered saline; RP-HPLC, reversed phase-high performance liquid chromatography; SUV, small unilamellar vesicles; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

* Correspondence to: Department of Applied Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki 852-8521, Japan.

Contract/grant sponsor: Ministry of Education, Science, Sports and Culture of Japan; Contract/grant number: 10680570

CCC 1075–2617/99/070298-08$17.50

showed strong membrane-perturbing activity and antibacterial activity against Gram-positive bacteria [18,19]. Furthermore, by use of its more hydrophilic and/or longer derivatives, e.g. Ac-(Leu-Ala-Arg-Leu)$_3$-(Leu-Arg-Ala-Leu)$_3$-NHCH$_3$ (**4$_6$**), we observed that such derivatives had no antibacterial and weak membrane-perturbing activities, whereas **4$_6$** exhibited a higher membrane-fusing activity than **4$_3$** [20]. The introduction of Pro, which is a typical helix breaker, into **4$_3$** would be anticipated to induce a reduction of α-helicity of **4$_3$**. The study on [Pro^6]**4$_3$**, [Pro^{2,6}]**4$_3$** and [Pro^{2,6,10}]**4$_3$** showed that the helical content gradually decreased in this order. However, [Pro^{2,6,10}]**4$_3$** still kept α-helix-like structures in the presence of acidic liposomes, meaning that Pro does not always behave as a strong helix breaker in specific surroundings [21].

In the present study, we intended to study the effects of changing α-helix propensity, hydrophobicity and basicity in **4$_3$** on its secondary structure and activity toward phospholipids. Therefore, we synthesized nine **4$_3$** analogs that contain Gly or Val instead of Ala, or Gln instead of Arg in **4$_3$** (Table 1) and examined their secondary structures and properties by CD and membrane perturbation measurements, as well as hemolytic and antibiotic activity assays.

MATERIALS AND METHODS

General

Reagents used for the synthesis and analysis were of reagent grade. Amino acid derivatives and the resin were purchased from Watanabe Chemical (Hiroshima, Japan). Calcein was purchased from Dojindo (Kumamoto, Japan), and DPPC and DPPG were from Sigma (St. Louis, MO). Fluorescent data were taken on a Hitachi spectrophotometer F-3010 with a thermostatted cell holder.

Peptide Synthesis

Peptides were manually synthesized by the stepwise elongation of Boc-amino acid on oxime resin (0.5 mmol HO/g resin) as reported previously [20]. The Boc-amino acid derivatives used are as follows: Ala, Arg (Tos), Gln, Gly, Leu and Val. Boc-Leu (1 mmol) was reacted with the resin (1 g) by the HBTU-HOBt method to give Boc-Leu-oxime resin (0.52 mmol/g). The coupling protocol was as follows: (1) wash, DCM ($\times$ 1), (2) wash, 25% TFA/DCM ($\times$ 1), (3) deprotection, 25% TFA/DCM (30 min), (4) wash, DCM ($\times$ 2), (5) wash, *i*-PrOH ($\times$ 1), (6) wash, DCM ($\times$ 3), (7) wash, DMF ($\times$ 1), (8) coupling, Boc-amino acid (three equivalents), HBTU (three equivalents), HOBt (three equivalents), and DIEA (five equivalents) (30 min), (9) wash, DMF ($\times$ 3), (10) wash, DCM ($\times$ 2), and (11) Kaiser test. When the coupling was incomplete, the protocol was repeated from (6). After building up the peptide, the Boc group was removed, and the peptide was acetylated with Ac_2O (five equivalents) and DIEA (two equivalents) in DMC (10 ml/g resin) at 25°C for 30 min followed by treatment with CH_3NH_3Cl (five equivalents), AcOH (five equivalents) and DIEA (five equivalents) in DMF (15 ml/g resin) at 25°C for 24 h. The protecting groups of the side-chains were removed with anhydrous HF (10 ml) in the presence of anisole (0.5 ml) at 0°C for 60 min. The crude peptides were purified by RP-HPLC using Wakosil 5C18 column (A = 100% H_2O/0.1% TFA, B = 100% CH_3CN/0.1% TFA) or Wakosil 5C4 column (A = 100% H_2O/0.1% TFA, B = 100% CH_3CN/0.1% TFA:100% *i*-PrOH/0.1% TFA (1:1)). The peptide gave satisfactory results on FAB-MS and amino acid analysis (Table 2).

Table 1 Sequence of 12-mer Model Peptides

Peptide	Sequence
4$_3$	Ac-LeuAlaArgLeu-LeuAlaArgLeu-LeuAlaArgLeu-NHCH$_3$
G1	Ac-LeuAlaArgLeu-Leu**Gly**ArgLeu-LeuAlaArgLeu-NHCH$_3$
G2	Ac-Leu**Gly**ArgLeu-Leu**Gly**ArgLeu-LeuAlaArgLeu-NHCH$_3$
G3	Ac-Leu**Gly**ArgLeu-Leu**Gly**ArgLeu-Leu**Gly**ArgLeu-NHCH$_3$
V1	Ac-LeuAlaArgLeu-Leu**Val**ArgLeu-LeuAlaArgLeu-NHCH$_3$
V2	Ac-Leu**Val**ArgLeu-Leu**Val**ArgLeu-LeuAlaArgLeu-NHCH$_3$
V3	Ac-Leu**Val**ArgLeu-Leu**Val**ArgLeu-Leu**Val**ArgLeu-NHCH$_3$
Q1	Ac-LeuAlaArgLeu-LeuAla**Gln**Leu-LeuAlaArgLeu-NHCH$_3$
Q2	Ac-LeuAla**Gln**Leu-LeuAla**Gln**Leu-LeuAlaArgLeu-NHCH$_3$
Q3	Ac-LeuAla**Gln**Leu-LeuAla**Gln**Leu-LeuAla**Gln**Leu-NHCH$_3$

Table 2 Physicochemical Properties of Peptides

Peptide	$[MH]^+$	Amino acid ratio in aeid hydrolysis	Hydrophobicity[a]	α-Helical propensity[b]	β-Structural propensity[b]
4_3	1433	Ala 3.08(3), Leu 5.99(6), Arg 2.87(3)	−0.123	1.21	1.09
G1	1419	Gly 1.04(1), Ala 2.00(2), Leu 6.13(6), Arg 2.90(3)	−0.130	1.13	1.08
G2	1405	Gly 1.97(2), Ala 1.02(1), Leu 6.09(6), Arg 2.90(3)	−0.138	1.06	1.08
G3	1391	Gly 2.89(3), Leu 6.03(6), Arg 3.13(3)	−0.145	0.99	1.07
V1	1461	Ala 2.04(2), Val 1.07(1), Leu 5.88(6), Arg 3.03(3)	−0.098	1.18	1.16
V2	1489	Ala 1.01(1), Val 2.08(2), Leu 6.11(6), Arg 2.87(3)	−0.074	1.15	1.24
V3	1517	Val 3.15(3), Leu 5.93(6), Arg 2.88(3)	−0.050	1.12	1.31
Q1	1404	Glu 0.97(1), Ala 3.11(3), Leu 6.08(6), Arg 1.86(2)	−0.030	1.22	1.10
Q2	1375	Glu 1.89(2), Ala 3.00(3), Leu 6.15(6), Arg 0.92(1)	0.063	1.23	1.12
Q3	1336	Glu 2.99(3), Ala 3.12(3), Leu 6.11(6)	0.155	1.24	1.13

[a] Hydrophobicity was calculated using the consensus value of hydrophobicity value for each amino acid residue [23].
[b] α-Helical propensity and β-structural propensity were calculated according to the literature [24].

Preparation of Phospholipid Liposomes

SUV of DPPC and DPPC:DPPG (3:1) were prepared for CD and leakage experiments. Phospholipid (7.4 mg) was dissolved in $CHCl_3$:MeOH (3:1, 2 ml) and then dried by a stream of N_2 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 Taitec Ultrasonic Processor VP-5T at 10 W intensity. The vesicles were left for 30 min at 25°C before the measurements. Lipid concentration was 1 mM. The vesicles trapping calcein were prepared by the reported procedure [3]. The dried lipid (20 mg) was hydrated in the buffer (pH 7.4, 2.0 ml) containing 0.1 M calcein. The calcein-entrapped vesicles were separated from free calcein 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 20 mM Tris–HCl buffer solution (pH 7.4) or in the presence of 0.9 mM phospholipid vesicles. Measurements were done at 25 or 50°C. The CD data were calculated by the method by Scholtz *et al.* [22].

Calcein Leakage

To a 20 mM Tris–HCl buffer solution (pH 7.4, 2 ml) in a cuvette was added 20 μl of the vesicles containing 0.1 M calcein to give a vesicle solution with a final concentration of 70 μM. To the cuvette placed in a holder (25 or 50°C) was added 20 μl of an appropriate dilution of the peptides in the buffer. The fluorescent intensities of calcein were monitored at 515 nm (exited at 495 nm) and measured 2 min after the addition of the peptides. To measure the fluorescent intensity for 100% dye release, 10 μl of Triton X-100 (10% in water) was added to dissolve the vesicles. The percentage of the calcein release caused by the peptides was calculated by the equation, $100 \times (F - F_0)/(F_t - F_0)$, where F is the fluorescent intensity achieved by the peptides, F_0 and F_t are intensities observed without the peptide and after Triton X-100 treatment, respectively.

Hemolytic and Antibacterial Assays

To fresh rabbit blood (1 ml) PBS (pH 7.4, 1 ml) was added, the resulting mixture was centrifuged at 2600 rpm for 5 min and the precipitates were collected. Washings with PBS were repeated three times. The precipitates obtained were suspended with fourfold volumes of PBS. Hemolytic assay was carried out by the procedure of Argiolas and Pisano [25]. To a rabbit erythrocyte solution (5 μl), PBS solution (pH 7.4, 1 ml) was added followed by the peptides. The resulting suspension was incubated for 20 min at 37°C and then centrifuged. The supernatant was monitored at 413 nm. Since **Q3** was hardly dissolved in PBS solution, the assay could not be done. On the antibacterial assay, the minimum inhibitory concentration was measured by the standard agar dilution method as described previously [19]. In this case, **V3** and **Q3** were slightly soluble in Mueller Hinton medium. Therefore, **V3** and **Q3** were at first dissolved in small amounts of formic acid and MeOH, respectively, and diluted

with the medium. The solutions were used for the assay.

RESULTS AND DISCUSSION

Peptide Design and Synthesis

Our previous study showed that [Pro^{2,6,10}]**4$_3$** took a slightly α-helix-like structure in the presence of DPPC/DPPG (3/1) liposomes [21]. Furthermore, Deber and Li reported that helical propensity of peptides highly depends on environment [15]. On the basis of these findings, we attempted to study the effect of amino acid substitution in **4$_3$** on its secondary structure and property. The synthetic peptides are shown in Table 1. Gly and Val instead of Ala were introduced into **4$_3$**, because their helix propensities are low and the hydrophobicities of Gly and Val are lower and higher, respectively, than the hydrophobicity of Ala. Among uncharged amino acids, Gln has the helix propensity fairly similar to that of Arg (Table 2). Accordingly, replacement of Arg by Gln was done to evaluate the influence of charge. The *N*- and *C*-termini were masked by acetyl and methylamide groups, respectively, to avoid the influence of undesirable charge.

Peptide synthesis was carried out by a solid phase method using oxime resin, which is useful for preparation of protected peptides of medium size [26]. All the peptides were obtained in moderate yield.

CD Study

In order to elucidate the structural characteristics of the peptides, the CD measurements were carried out in Tris–HCl buffer solution (pH 7.4), 2,2,2-trifluoroethanol (TFE) and the buffer solution containing phospholipid liposomes. The results are shown in Figure 1 and α-helical contents calculated according to the literature by Scholtz *et al.* [22] are listed in Table 3. In the buffer (Figure 1A1 and A2), **G1**, **G2** and **G3** were random, and Gln-containing peptides showed higher helical contents than **4$_3$**. **V1** exhibited low helicity of 18%, whereas **V2** and **V3** did not give an α-helix pattern but rather a β-structure. These results are reasonable from the values of α-helical and β-structural propensities in Table 2. High temperature (50°C) in the buffer induced a decrease in helical content, indicating some loss of α-helical structure. Peptide **4$_3$** had the helical content of 44% in TFE at 25°C. On Gln-containing peptides, the contents slightly increased in the order of **Q1** < **Q2** < **Q3** (Table 3 and Figure 1B2). On the other hand, the helical contents decreased with an increase in the number of Gly in the case of Gly-containing peptides (Figure 1B1). It is interesting that **V1**, **V2** and **V3** exhibited moderate helical contents in TFE (Table 3), supporting the viewpoint by Deber and Li [15].

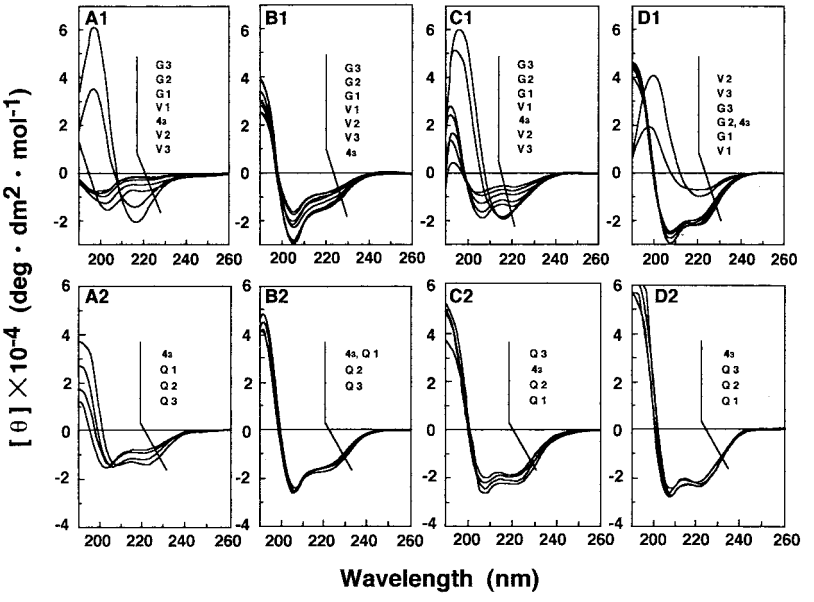

Figure 1 CD spectra of the peptides in 20 mM Tris–HCl buffer (pH 7.4) (A1, A2), TFE (B1, B2), DPPC (C1, C2) and DPPC:DPPG (3:1) vesicles (D1, D2) at 25°C; [peptide] = 5.0 μM, [lipid] = 0.9 mM.

Table 3 α-Helical Contents of Peptides

Peptide	α-Helical content (%)[a]						
	Buffer (pH 7.4)		TFE	DPPC		DPPC:DPPG (3:1)	
	25°C	50°C	25°C	25°C	50°C	25°C	50°C
4_3	24	R	44	50	46	55	52
G1	R	R	31	34	31	62	62
G2	R	R	26	27	26	55	49
G3	R	R	24	20	21	51	50
V1	18	R	33	46	48	64	61
V2	β	β	37	β	β	20	20
V3	β	β	43	β	β	β	β
g1	27	22	44	61	51	67	57
g2	34	27	47	59	50	65	54
g3	40	37	50	49	31	59	49

[a] β and R denote β-structure and random, respectively.

In the presence of neutral DPPC vesicles (Figure 1C1 and C2), the helical contents of Gly-containing peptides were roughly parallel to those in TFE. Although **V1** could form α-helices, **V2** and **V3** again gave β-structure, indicating the strong helix-breaking property of Val under the conditions used. The fact that a rise in temperature gave no appreciable conformational change for Gly-containing peptides and **V1** may indicate that these peptides form stable conformation. In contrast, the helical contents of **g1**, **g2** and **g3** decreased with a rise in temperature to cause a probable loss of the peptide structure. A similar tendency was observed in the presence of acidic DPPC:DPPG (3:1) vesicles (Figure 1D1 and D2). Higher content values suggest that the electrostatic interaction between basic peptide and acidic phospholipids may help the helix formation. The CD curve that is probably attributable to a mixture of α-helix (20%), β-structure (40%) and random (40%) was found for **V2** in acidic liposomes. This means that an Ala→Val substitution in **4_3** is allowed to form α-helical structure, but two substitutions are on the boundary (Table 3).

Calcein Leakage

The results are shown in Figure 2. In the presence of DPPC vesicles at 25°C (Figure 2A), **V1** showed exceptionally strong leakage activity among the Gly- and Val-containing peptides. This suggested the importance of hydrophobicity for the peptide–lipid binding. Indeed the leakage activity of **G1**, **G2** and **G3** decreased in this order. In addition to hydrophobicity, amphiphilicity is also required for strong leakage activity. Although **V2** and **V3** have higher hydrophobicity than **V1**, they showed very weak leakage activity probably due to incomplete amphiphilicity: when Val-containing peptides form a β-structure, hydrophobic Leu and Val residues are present on one side and hydrophobic Leu and hydrophilic Arg residues on another side along the peptide chain. As the result, the peptides have in-

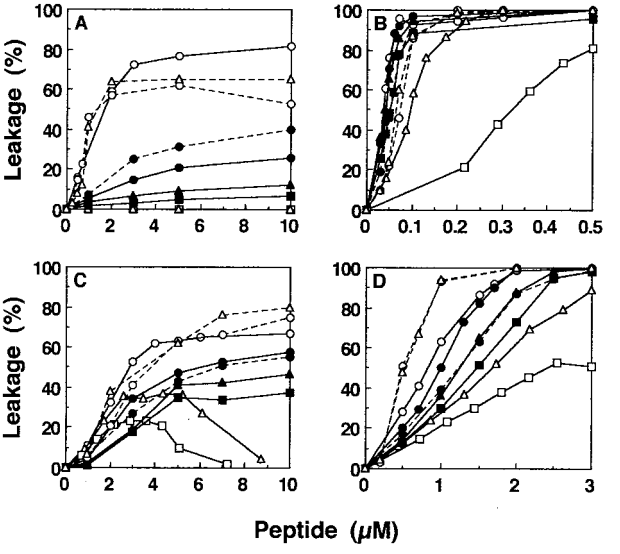

Figure 2 Profiles of the calcein leakage as a function of the peptide concentration. (A) DPPC at 25°C, (B) DPPC at 50°C, (C) DPPC:DPPG (3:1) at 25°C and (D) DPPC:DPPG (3:1) at 50°C; [lipid] = 70 μM. **4_3** (--●--), **G1** (— ● —), **G2** (— ▲ —), **G3** (— ■ —), **V1** (— ○ —), **V2** (— △ —), **V3** (— □ —), **g1** (--○--) and **g2** (--△--).

complete amphiphilic structure. **Q1** and **Q2**, which have higher hydrophobicity than $\mathbf{4_3}$, exhibited the maximum activity of 60–65%, which was stronger than that of $\mathbf{4_3}$. In the presence of DPPC vesicles at 50°C (Figure 2B), the peptides except for **V2** and **V3** showed the maximum leakage activity of ca. 100% within or at the peptide concentration of 0.2 μM, suggesting that the structure of DPPC membrane becomes unstable above the phase transition temperature and the increase in fluidity of lipid membrane may allow the easy penetration of peptide into the hydrophobic region of lipid.

In the presence of acidic DPPC:DPPG (3:1) vesicles at 25°C (Figure 2C), most peptides revealed slightly stronger leakage activity as compared with neutral DPPC vesicles. It is likely that the electrostatic interaction of basic peptides with acidic lipid membrane increased the population of the bound peptides to cause destabilization of the membrane [20]. Under the same conditions, **V2** and **V3** exhibited the maximum activity at the peptide concentration of 2.5–4 μM and then the activity gradually decreased. This phenomenon may be due to an incident other than dye leakage. At 50°C (Figure 2D), all the peptides showed much stronger leakage activities than those observed at 25°C. Complete leakage was observed for the peptides other than **V2** and **V3** at the peptide concentration of 2–3 μM. However, the peptide concentrations required for complete leakage at 50°C were different between the cases in acidic vesicles (2–3 μM) and in neutral vesicles (0.2 μM). This result may be interpreted by prevention of peptide penetration into the hydrophobic region of lipid membrane because of electrostatic interaction between charged peptides and lipids at the surface of membrane [27].

Hemolytic and Antibacterial Activity

Hemolytic activity was measured using rabbit erythrocytes in order to evaluate the effect of the peptides on cell membrane. The results are shown in Figure 3. Peptide $\mathbf{4_3}$ showed the maximum activity at the peptide concentration of 9 μM. The activity of **G1**, **G2** and **G3** was found to be lower than that of $\mathbf{4_3}$, whereas **V1**, **V2** and **V3** had higher activity. It is interesting that **V2** and **V3** induced strong hemolysis. Peptides **V2** and **V3** have incomplete amphiphilicity but high hydrophobicity, suggesting that hydrophobicity of peptides exceeds their amphiphilicity for hemolytic activity. However, the reverse was found for Gln-containing peptides, which showed a weaker activity than that of $\mathbf{4_3}$ in spite of having higher hydrophobicity. Although the reason is unclear, decrease in charged Arg residue(s) may weaken the interaction of the peptides with rabbit red blood cell membrane.

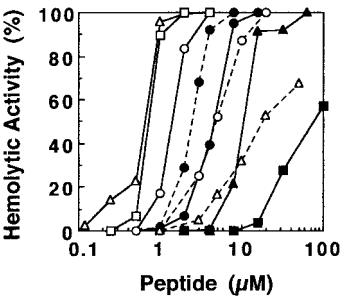

Figure 3 Profiles of the hemolysis as a function of the peptide concentration. $\mathbf{4_3}$ (--●--), **G1** (– ● –), **G2** (– ▲ –), **G3** (– ■ –), **V1** (– ○ –), **V2** (– △ –), **V3** (– □ –), **Q1** (--○--) and **Q2** (--△--).

The antibacterial activities of peptides are listed in Table 4. The activity against various Gram-positive bacteria was observed for $\mathbf{4_3}$ and the related peptides except for **V3** and **Q3**. On the other hand, none of the peptides were effective against Gram-negative bacteria even at the peptide concentration of 50 μg/ml. It is noticeable that **G1**, **G2** and **G3** exhibited the same activity as that of $\mathbf{4_3}$, suggesting that amphiphilicity is more important than hydrophobicity for the antibacterial activity against Gram-posive bacteria. The fact that **V1**, **Q1** and **Q2** have the same activity also support this view. Since bacterial membrane contains acidic phospholipids, **V2** may partially take an α-helical structure by interaction with the membrane to show the activity. The lack of antibacterial activity of **V3** and **Q3** indicates that amphiphilicity and the presence of basic amino acid residue are required for the activity. From the study on the activity of four α-helical antimicrobial peptides based on sequences and amphiphilicity of many naturally occurring peptides having α-helical domains, Tossi *et al.* found that the peptides with the side-chain projection angle of ca. 100° and cationic residues exhibited high bioactivity against Gram-positive and -negative bacteria [9]. Our data are in accord with their results.

CONCLUSIONS

The results showed that more than two Ala→Val substitutions in $\mathbf{4_3}$ were not enough to holding its α-helical structure, while the Ala→Gly or Arg→Gln

Table 4 Antibacterial Activity of Peptides

Organism	Minimum inhibitory concentration (μg/ml)[a]									
	4_3	**G1**	**G2**	**G3**	**V1**	**V2**	**V3**	**Q1**	**Q2**	**Q3**
Staphylococcus aureus FDA 209p	6.25	6.25	3.13	3.13	6.25	12.5	>50	3.13	6.25	>50
S. aureus 1840	6.25	6.25	6.25	6.25	6.25	12.5	>50	3.13	6.25	>50
S. epidermidis ATCC 12228	6.25	6.25	6.25	6.25	6.25	12.5	>50	6.25	6.25	>50
Streptococcus pyrogenes C-203[b]	25	25	25	25	50	>50	>50	25	25	>50
Enterococcus faecalis LS-101[b]	6.25	6.25	6.25	6.25	12.5	50	>50	12.5	25	>50
Bacillus subtilis PCI 219	6.25	6.25	6.25	6.25	6.25	12.5	>50	6.25	6.25	>50
Escherichia coli NIHJ JC-2	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
Shigella flexneri EW-10	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
Proteus vulgaris IFO 3988	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50

[a] Method: agar dilution method; Medium: Mueller Hinton medium (Difco); Inoculum size, 10^6 cells/ml.
[b] Added 10% horse blood.

substitution gave little structural change. High hydrophobicity and helical propensity were favorable for strong membrane-perturbing activity. Furthermore, hydrophobicity was likely to be more important than amphiphilicity for hemolytic activity, but the opposite situation was observed for antibacterial activity. The present study exhibited that the helix-breaking ability of Val in **4_3** was unexpectedly stronger as compared with that of Pro under the conditions tested [21], although it has been found that Pro has the lowest helix propensity in the membrane-mimic environment of SDS micelles [15].

Acknowledgements

This work was supported by a Grant-in-Aid (No. 10680570) from the Ministry of Education, Science, Sports and Culture of Japan. The authors would like to thank Drs Y. Ikeda and M. Miyoshi, Yoshitomi Pharmaceutical Industries, Ltd. for the antibiotic assay and Mr K. Yoshida and Mr Y. Mukai in this laboratory for their technical assistance.

REFERENCES

1. Tytler EM, Anantharamaiah GM, Walker DE, Mishra VK, Palgunachari MN, Segrest JP. Molecular basis for prokaryotic specificity of magainin-induced lysis. *Biochemistry* 1995; **34**: 4393–4401.
2. Wieprecht T, Dathe M, Epand RM, Beyerman M, Krause E, Maloy WL, MacDonald DL, Bienert M. Influence of the angle subtended by the positive charged helix face on the membrane activity of amphipathic, antibacterial peptides. *Biochemistry* 1997; **36**: 12869–12880.
3. Sakamoto S, Mihara H, Matsuo E, Niidome T, Anzai K, Kirino Y, Aoyagi H. Enhanced membrane-perturbing activities of bundled amphiphilic α-helix polypeptides on interaction with phospholipid bilayer. *Bull Chem Soc Jpn* 1995; **68**: 2931–2939.
4. Saberwal G, Nararaj R. Cell-lytic and antibacterial peptides that act by perturbing the barrier function of membranes: facets of their conformational features, structure-function correlations and membrane-perturbing abilities. *Biochim Biophys Acta* 1994; **1197**: 109–131.
5. Colotto A, Martin I, Ruysschaert J-M, Sen A, Hui SW, Epand RM. Structural study of the interaction between the SIV fusion peptide and model membranes. *Biochemistry* 1996; **35**: 980–989.
6. Ishiguro R, Matsumoto M, Takahashi S. Interaction of fusogenic synthetic peptide with phospholipid bilayers: orientation of the peptide α-helix and binding isotherm. *Biochemistry* 1996; **35**: 4976–4983.
7. Pécheur E-I, Martin I, Ruysschaert J-M, Bienvenüe A, Hoekstra D. Membrane fusion induced by 11-mer anionic and cationic peptides: a structure–function study. *Biochemistry* 1998; **37**: 2361–2371.
8. Hancock RE. Peptide antibiotics. *Lancet* 1997; **349**: 418–422.
9. Tossi A, Tarantio C, Romeo D. Design of synthetic antimicrobial peptides based on sequence analogy and amphipathicity. *Eur J Biochem* 1997; **250**, 594–558.
10. Blondelle SE, Houghten RA. Design of model amphipathic peptides having potent antimicrobial activities. *Biochemistry* 1992; **31**: 12688–12694.
11. Matsuzaki K, Murase O, Fujii N, Miyajima K. Translation of a channel-forming antimicrobial peptide, magainin 2, across lipid bilayers by forming a pore. *Biochemistry* 1996; **34**: 6521–6526.
12. Marsh D. Peptide models for membrane channels. *Biochem J* 1996; **315**: 345–361.

13. Peled-Zehavi S, Arkin IT, Engelman DM, Shai Y. Coassembly of synthetic segments of Shaker K^+ channel within phospholipid membranes. *Biochemistry* 1996; **35**: 6828–6838.
14. Futaki S, Aoki M, Fukuda M, Kondo F, Niwa M, Kitagawa K. Assembly of the four individual helices corresponding to the transmembrane segments (S4 in repeat I–IV) of the sodium channel. *Tetrahedron Lett* 1997; **38**: 7071–7074.
15. Deber CM, Li S-C. Peptides in membranes: helicity and hydrophobicity. *Biopolymers* 1995; **37**: 295–318.
16. Liu LP, Li S-C, Goto NK, Deber CM. Threshold hydrophobicity dictates helical conformtions of peptides in membrane environments. *Biopolymers* 1996; **39**: 465–470.
17. Piela L, Nemethy G, Scheraga HA. Proline-induced constraints in α-helices. *Biopolymers* 1987; **26**: 1587–1600.
18. Lee S, Mihara H, Aoyagi H, Kato T, Izumiya N, Yamasaki N. Relationship between antimicrobial activity and amphiphilic property of basic model peptides. *Biochim Biophys Acta* 1986; **862**: 211–219.
19. Mihara H, Kanmera T, Yoshida M, Lee S, Aoyagi H, Kato T, Izumiya N. Design and synthesis of amphiphilic basic peptides with antibacterial activity and their interaction with model membrane. *Bull Chem Soc Jpn* 1987; **60**: 697–706.
20. Ohmori N, Niidome T, Hatakeyama T, Mihara H, Aoyagi H. Interaction of α-helical peptides with phospholipid membrane: effect of chain length and hydrophobicity of peptide. *J Peptide Res* 1998; **51**: 103–109.
21. Lee S, Park NG, Kato T, Aoyagi H, Kato T. Effect of proline in basic and α-helical amphipathic peptides on acidic liposomes and their antimicrobial activity. *Chem Lett* 1989, 599–602.
22. Scholtz JM, Qian H, York EJ, Stewart JM, Baldwin RL. Parameters of helix–coil transition theory for alanine-based peptides of varying chain lengths in water. *Biopolymers* 1991; **31**: 1463–1470.
23. Eisenberg D. Three-dimensional structure of membrane and surface proteins. *Annu Rev Biochem* 1984; **53**: 595–623.
24. Chou PY, Fasman GD. Empirical properties of protein conformation. *Annu Rev Biochem* 1978; **47**: 251–276.
25. Argiolas A, Pisano JJ. Bombolitins, a new class of mast cell degranulating peptides from the venom of the bumblebee *Megabombus pennsylvanicus*. *J Biol Chem* 1985; **260**: 1437–1444.
26. Kaiser ET, Mihara H, Laforet GA, Kelly JW, Walters L, Findeis MA, Sasaki T. Peptide and protein synthesis by segment synthesis-condensation. *Science* 1989; **243**: 187–192.
27. Dathe M, Shumann M, Wieprecht T, Winkler A, Beyerman M, Krause E, Matsuzaki K, Murase O, Bienert M. Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. *Biochemistry* 1996; **35**: 12612–12622.

