N- and C-Terminal Effect of Amphiphilic α-Helical Peptides on the Interaction with Model- and Bio-Membranes

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We previously designed and synthesized five N- and C-termini-free amphiphilic α-helical model peptides (Hel series) with a systematically varied hydrophobic–hydrophilic balance (HHB) that showed hemolytic activity, but no antimicrobial activity. However, an N-acetylated and C-amidated model peptide, peptide 3 [S. E. Blondelle and R. A. Houghten, *Biochemistry*, **31**, 12688 (1992)], similar to a Hel series peptide, Hel 9-9, whose hydrophobic and hydrophilic amino acid residues and areas are equal in the α-helical structure, have exhibited both hemolytic and antimicrobial activities. Thus, to investigate the N- and C-terminal effect of the Hel series peptides on their antimicrobial activity, we designed and synthesized three peptides (Cap-Hel series), both termini-blocked by N-acetyl and C-amide groups. Their interaction mode with membranes was examined through reverse-phase high-performance liquid chromatography and circular dichroism spectroscopy as well as measurements of the hemolytic activity, antimicrobial activity, and membrane-clearing ability. No essential difference was found in either the terminal-free or -protected peptides, indicating that acetylation of the N-terminal and amidation of C-terminal did not affect their intrinsic antimicrobial activity in spite of a considerable change in the binding properties to lipids and hemolytic activities.

The cytotoxic amphiphilic α-helical peptides were widely found in natural sources, some of which function as cell-lytic agents and/or some of which function as antibacterial ones.[1—3] The cationic peptide antibiotics have recently been expected as drugs for such microorganisms that have resistance to well-known antibiotics: penicillin, ampicillin etc., because the action mechanism of the peptide antibiotics is entirely different from that of the other ones, which may not face bacterial resistance.[3,4] The specific feature of cationic peptides is as follow: They have both a hydrophobic face, comprising non-polar amino acid residues, and a polar hydrophilic face with positively charged residues. A recent study on the structure–activity of antimicrobial peptides has suggested that the combination of hydrophobicity and the net positive charge may be sufficient to exhibit antibacterial activity.[5]

In this connection, we have found that a balance produced by hydrophobic–hydrophilic faces of amphiphilic α-helices (hydrophobic–hydrophilic balance, HHB) can be correlated to peptide-membrane interactions and even to biological functions.[6] Model peptides (Hel series) consisting of 18-mer residues are made up of only three kinds of amino acids: hydrophilic Lys, hydrophobic Leu, and fluorescent Trp (Fig. 1). The Hel-series peptides increased hemolytic activity with increasing hydrophobicity, but showed no antimicrobial activity. Contrary to this, Blondelle et al. have reported that an amphiphilic α-helical model peptide (peptide 3 in Fig. 1), almost the same as Hel 9-9 in terms of HHB, had a slight hemolytic and a low, but distinct, antimicrobial activity.[7] There is little difference between Hel 9-9 and peptide 3, not only in the amino acid composition, but in the sequences, except that Hel 9-9 has Trp; however, both termini were free, while peptide 3 has N-acetylated and C-amidated terminal groups, but lacks Trp. On the other hand, upon considering the *de novo* design and synthesis of peptides having biological activity, we are often troubled by whether both termini should be free or protective.[2,7—12] Thus, to evaluate the significance of the terminal groups on the biological activity we synthesized Hel series-relating peptides having N-acetylated and C-amidated termini (Cap-Hel series). Their interaction mode with membranes was examined through reverse-phase high-performance liquid chromatography (RP-HPLC), circular dichroism (CD) spectroscopy, and measurements of the hemolytic activity, antimicrobial activity, and membrane clearing ability.

Experimental

Materials. 9-Fluorenylmethoxycarbonyl (Fmoc)-PAL-PEG-PS (polyethyleneglycol-polystyrene) resin was purchased from Japan PerSeptive Biosystems Ltd. (Tokyo, Japan). Fmoc amino acids, coupling reagents (1-hydroxybenzotriazole; HOBT and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TBTU), and a deprotecting agent (2,2,2-trifluoroaceteic acid; TFA) were purchased from Watanabe Chem. Ind., Ltd. (Hiroshima, Japan). Other reagents for peptide synthesis [*N*,*N*-dimethylformamide, *N*-ethyldiisopropylamine, acetic anhydride, piperidine, *p*-methoxybenzenethiol (thioanisole), 1,2-ethandithiol, and *m*-cresol] were obtained from Nacalai Tesque Co., Inc. (Kyoto, Japan). Egg-yolk phosphatidylcholine (egg PC) and egg-yolk phosphatidylglycerol (egg PG) were purchased from Sigma Chemical Co. (St. Louis, USA). 2-[tris(hydroxymethyl)methylamino]ethanesulfonic acid (TES) was obtained from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were purchased from Nacalai Tesque and

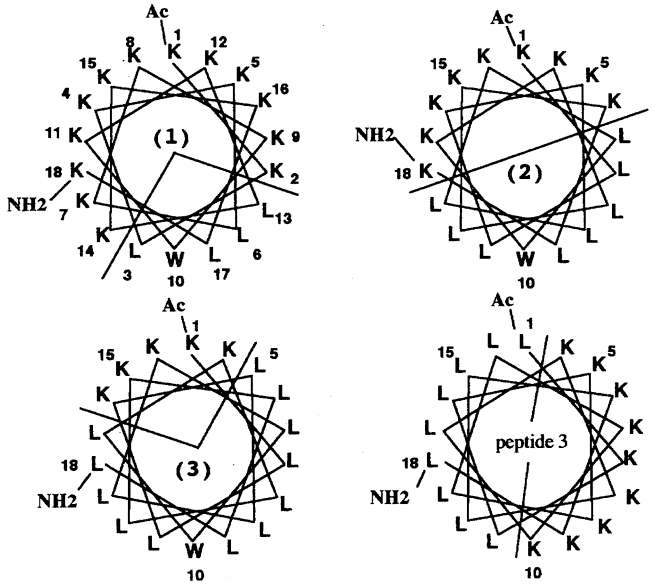
Wako Pure Chemicals (Osaka, Japan) in high analytical grade.

Synthesis of Model Amphiphilic Peptides. The primary structure of the peptides and their helical wheel representations are shown in Fig. 1. All peptides were synthesized by Fmoc chemistry based on a solid-phase technique starting with Fmoc-PAL-PEG-PS resin (0.6 g, 0.12 mmol scale) using a Milligen automatic solid-phase peptide synthesizer (Model 9050), as described previously.[6] The peptides were cleaved from the resin by treating with TFA (22.4 ml) containing thioanisole (3.5 ml), 1,2-ethanedithiol (1.8 ml), *m*-cresol (0.6 ml) for 2 h at room temperature. The crude peptides were purified by gel filtration on Sephadex G-25 (25×1300 mm). Furthermore, the peptides, except for Cap-Hel 13-5, were applied on a preparative C18-300 (20×250 mm, YMC) RP-HPLC column, while Cap-Hel 13-5 was applied on a preparative C8-300 (20×250 mm, YMC) RP-HPLC column with a linear A and B gradient at a flow rate of 5 ml min^{-1}, where solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. The yields of Cap-Hels 5-13, 9-9, and 13-5 obtained after purification were 70, 51, and 34 mg, respectively. The purity of the peptides was confirmed by RP-HPLC using an analytical C18-300 (4.6×250 mm) column, amino acid analysis, and mass-spectrum analysis. For the amino acid analysis, purified peptides were hydrolyzed in 6 M HCl containing 4% mercaptoacetic acid and 0.6% phenol at 110 °C for 24 h. A hydrolyzed peptide solution was applied on an amino acid analyzer (JASCO 851-AS). The amino acid analysis data of all peptides were in good agreement with theoretical ones with less than 5% error from the corresponding amino acid composition. Mass spectra were taken by a mass spectrometer (JEOL JMS-HX 110). Corresponding molecular ion peaks were obtained for the tree peptides. The peptide concentrations in solution were determined from UV-absorbance of Trp at 280 nm in a buffer containing 6 M guanidine hydrochloride (Gu·HCl) (1 M = 1 mol dm^{-3}).

Preparation of Liposome. A lipid (egg PC or egg PC/egg PG mixture) solution in chloroform/methanol was placed in a round-bottom flask. After drying in a stream of N_2 gas, the residual film was further dried under vacuum over night. After the lipid film was hydrated with a TES buffer (5 mM TES/100 mM NaCl, pH = 7.4), the suspension was vortexed for 20 min. This obtained turbid liposome solution was employed for membrane-clearing experiments. For the other experiments, the solution was sonicated in an ice bath with nitrogen flowing for 10 min (×3) by using a sonicater equipped with a titanium tip.

Liposome-Lytic Experiment. The turbid liposome solution prepared as described above was diluted to a concentration of about 100 μM with the same buffer (5 mM TES/100 mM NaCl, pH = 7.4). The peptide solutions were then added to the solution to attain a given mole ratio of peptide to lipid, and then incubated at 25 °C. The absorbance of the sample solution was recorded at 400 nm after vigorous vortexing at proper time intervals.

CD Spectrum Measurements. The CD spectra in 80% 2,2,2-



Hel 5-13 : H-KKLKKLKKKWKKLKKKLK-OH

Hel 9-9 : H-KLLKKLLKLWKKLLKKLK-OH

Hel 13-5 : H-KLLKLLLKLWLKLLKLLL-OH

(1) Cap-Hel 5-13 : Ac-KKLKKLKKKWKKLKKKLK-NH_2

(2) Cap-Hel 9-9 : Ac-KLLKKLLKLWKKLLKKLK-NH_2

(3) Cap-Hel 13-5 : Ac-KLLKLLLKLWLKLLKLLL-NH_2

peptide 3 : Ac-LKLLKKLLKKLKKLLKKL-NH_2

Fig. 1. Primary structure of model peptides and their helical wheel representation.

trifluoroethanol (TFE) and in the absence and presence of neutral or acidic liposomes were recorded on a JASCO J-600 instrument with a personal computer (NEC PC-9801). All measurements were carried out at 25 °C. In general, the peptide and lipid concentrations were about 20 and 550 μM. The lipid ratio of Egg PC/Egg PG was 3/1. In the case of Cap-Hel 5-13, the peptide and lipid concentrations were 10 and 1000 μM, respectively, and the lipid ratio of Egg PC/Egg PG was 7/1. The α-helical contents (f_h) were calculated from the following equation:

$$f_h = ([\theta]_{222} - [\theta]^0_{222})/[\theta]^{100}_{222},$$

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm. The values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, corresponding to 0 and 100% helical contents at 222 nm, are estimated to be −2000 and −30000 deg cm^2 dmol^{-1}, respectively.[12,13]

Hemolytic Assays. The hemolytic activities of the peptides were determined using human blood red cells (RBCs). The RBCs were collected from citric acid-treated blood by centrifugation (at 2500 rpm for 5 min) and washed four times with phosphate buffer saline (PBS: 10 mM phosphate buffer/150 mM NaCl, pH=7.4) to remove any plasma and buffy coat. Suspensions of 0.3% hematocrit (2×10^7/ml) in PBS with or without a peptide were incubated for 90 min at 25 °C. Hemolysis was expressed as the hemoglobin content (absorbance at 542 nm) of the supernatant after centrifugation at 2500 rpm for 5 min. A 100% hemolysis was determined by hemoglobin release after the addition of 0.3% Triton X-100.

Antimicrobial Assay. The minimum inhibitory concentration (MIC) of the growth of microorganisms was determined by the standard agar dilution method using Muellar Hinton agar (Difco), as done previously.[23] The final bacterial suspension held 10^6 colony-forming units (CFU)/ml.

Results

Design of Peptides. The primary structures of the Cap-Hel series and their helical representations are shown in Fig. 1. In a previous study we could classify five amphiphilic α-helical peptides with a set of various hydrophobic–hydrophilic balances into three categories based on their action mode for model and bio-membranes: The peptides with a hydrophobic face larger than the hydrophilic face (Hels 13-5 and 11-7), the inverse ones (Hels 5-13 and 7-11) and the peptide with a same hydrophobic–hydrophilic face (Hel 9-9).[6] Thus, in the present study we selected three peptides (Cap-Hels 13-5, 9-9, 5-13) belonging to the respective categories.

RP-HPLC. To determine the quantitative HHB of amphiphilic substances, RP-HPLC has often been employed.[7,15,19] In the present RP-HPLC experiments, the peptides of the Cap-Hel series were eluted in the order of increasing hydrophobic regions in a similar manner as those of the Hel series (Table 1). It is noted that the peptides of the Cap-Hel series were eluted slightly later than the corresponding peptides of the Hel series, indicating that the blocking (capping) of both terminals increases the hydrophobicity of the peptides, as expected.

CD Measurement. The secondary structure was determined by CD measurements in various media (Fig. 2). In TFE, a strong helix-inducing solvent, all peptides showed double-minimum CD curves at around 208 and 222 nm, i.e., a characteristic of the α-helical structure. As for the obtained helical contents, the CD spectra showed that the Cap-Hel series peptides are about 1.5-times larger than the Hel-series peptides (Table 1). However, in a buffer solution (5 mM TES/100 mM NaCl, pH=7.4) with a peptide concentration of 20 μM, and in the presence of egg PC and egg PC/egg PG, these peptides took considerably different conformations, as follows. Cap-Hel 5-13 took a random structure, except for only taking a slightly α-helical structure (10%) in the presence of acidic liposomes, while Cap-Hel 9-9 took a random structure in a buffer. However, upon the addition of neutral and acidic liposomes, its helical contents increased in the

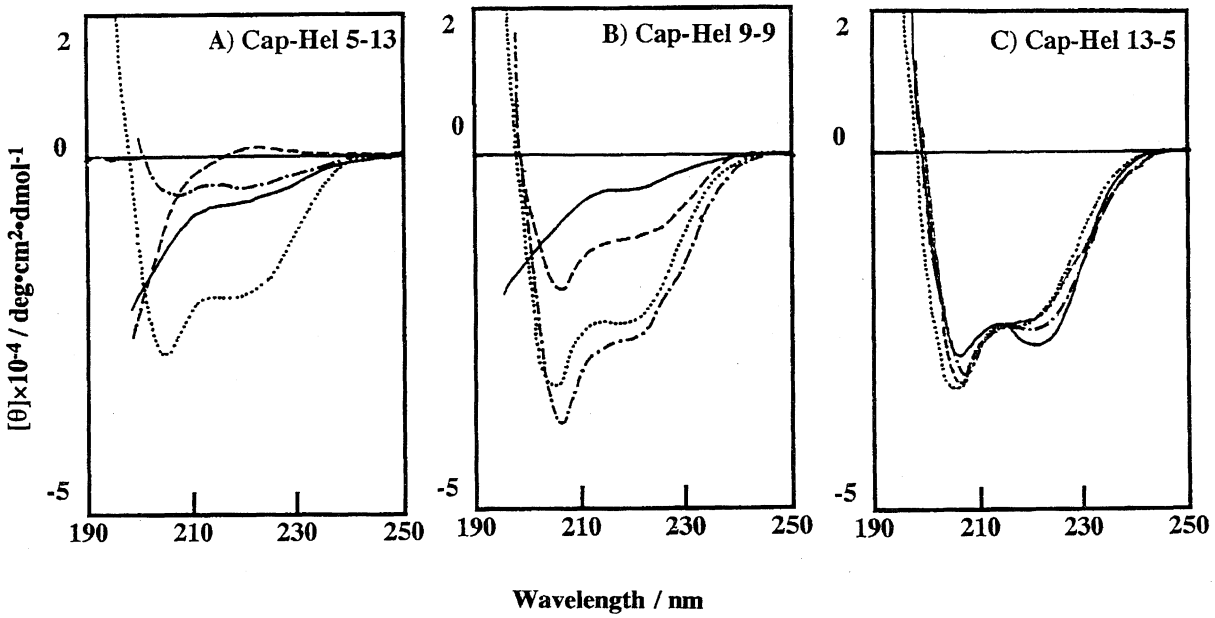


Fig. 2. Circular dichroism spectra of Cap-Hel series peptides; Cap-Hel 5-13 (A), Cap-Hel 9-9 (B), and Cap-Hel 13-5 (C). 80% TFE (····); TES buffer (——); Egg PC (- - -); egg PC–egg PG (3 : 1) (–·——·–).

Table 1. Retention Times and α-Helical Contents of Amphiphilic Model Peptides in Various Media

	R.T.[a]	α-Helical contents			
		TES buffer	80% TFE	egg PC[b]	egg PC/egg PG
Hel 5-13[e]	23.8	0	0.34	0	0.12[c]
Hel 9-9[e]	35.0	0.03	0.41	0.22	0.45[d]
Hel 13-5[e]	50.2	0.68	0.45	0.75	1.00[d]
Cap-Hel 5-13	26.3	0	0.59	0	0.10[c]
Cap-Hel 9-9	40.0	0.13	0.70	0.40	0.66[d]
Cap-Hel 13-5	56.5	0.80	0.72	0.75	0.79[d]

a) RP-HPLC retention times were determined using a C18 column (4.8×250 mm). Peptide elution was monitored at 280 nm using a gradient system consisting of 0.1% TFA–acetonitorile–H_2O. b) Molar ratio of peptides and lipid, [P]/[L], was 1 : 25. c) The ratio of peptides and egg PC/egg PG (7/1) was 1 : 100. d) The ratio of peptides and egg PC/egg PG (3/1) was 1 : 25. e) Kiyota et al.[6]

order. In contrast, Cap-Hel 13-5 took an α-helical structure with almost the same helical contents in all media. It is noted that in the presence of neutral and acidic liposomes the helical contents of Cap-Hels 5-13 and 9-9 are generally increased, as compared with the corresponding Hel peptides, but that of Cap-Hel 13-5 is not changed, suggesting that Cap-Hel 13-5 can take a stable amphiphilic structure, regardless of the surroundings.

Liposome-Lytic Power of Model Peptides. The interactions of the peptides with neutral and acidic lipid bilayers were monitored by the turbidity change of the liposome with time. The vigorously vortexed liposome suspensions were incubated with and without the peptides at 25 °C (Fig. 3). Upon the addition of the peptide, distinct differences were observed in acidic and neutral liposomes. In the presence of egg PC liposomes, the absorbance for Cap-Hels 9-9 and 13-5 gradually decreased within 10 h, and then attained a plateau, while no absorbance change was observed for Cap-Hel 5-13. In a clearing test of the egg PC liposomes, Cap-Hel 9-9 was slightly more effective compared with Hel 9-9, though the clearing power of Cap-Hel 13-5 was much less than that of Hel 13-5 (see Fig. 3A). Hel series peptides showed an increase in the clearing power with increasing hydrophobicity; similarly, the power of the Cap-Hel series also followed the order of hydrophobicity magnitude. However, it should be noted that Cap-Hel 13-5 has a weaker clearing power compared with Hel 13-5 (with no cap). This is in contrast to the general concept that the higher is the hydrophobicity, the stronger is the clearing power. On the other hand, both Cap-Hel 5-13 and Hel 5-13 are poor at lipid clearing. This is compatible with the results of the CD experiments from which these peptides were found to take a weakly or scarcely α-helical structure in the presence of neutral liposomes.

In acidic liposomes comprising egg PC–egg PG (3 : 1), the addition of each peptide induced a rapid change in the absorbance in an increasing or decreasing manner. Cap-Hel 9-9 showed almost the same effect on clearing the liposome-turbidity, as did Hel 9-9 (see Fig. 3B). The addition of Cap-Hel 5-13 to the liposomes resulted in a rapid increase in turbidity, which then started to decrease with time until 6 h

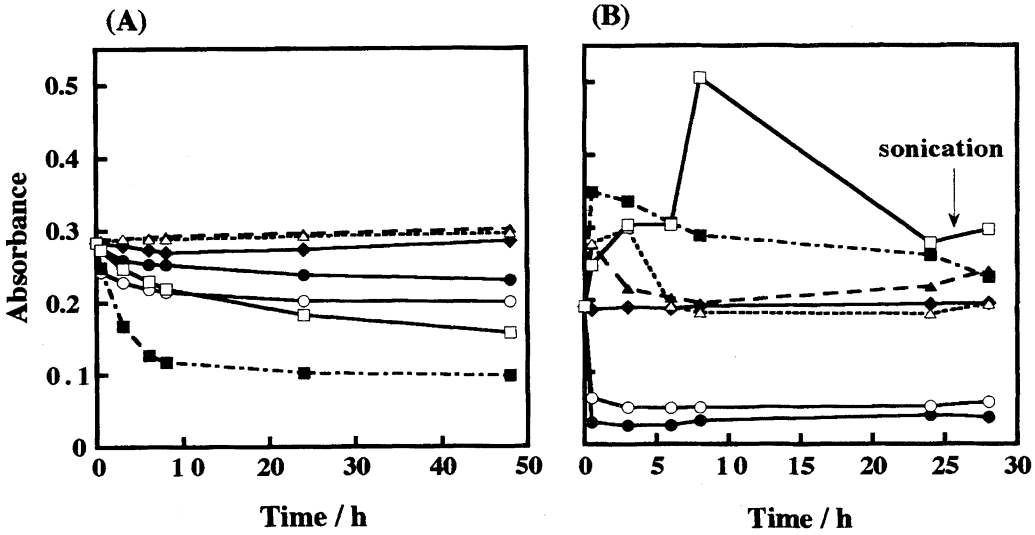


Fig. 3. The time-changes in absorbance showing the turbid liposome-clearing ability of Cap-Hel peptides and of the corresponding Hel peptides in egg PC liposomes (A) and egg PC–egg PG (3 : 1) liposomes (B). The symbols are as follows: (◆) control, (▲) Hel 5-13, (△) Cap-Hel 5-13, (●) Hel 9-9, (○) Cap-Hel 9-9, (■) Hel 13-5, (□) Cap-Hel 13-5. Petides and lipid concentrations were 20 μM and 100 μM respectivity for (A) and (B).

had passed; finally, it reached a plateau while Cap-Hel 13-5 underwent a rapid and then drastic and gradual increase up to 10 h; then, there was a gradual decrease with time. Here, it is of interest to note that both Cap-Hel 13-5 and Hel 13-5 made neutral liposomes clear. In contrast, when mixed with the acidic liposomes, these made it turbid. As for Cap-Hel 9-9, the clearing power is stronger than Hel 9-9 in neutral liposomes, but not in acidic liposomes. Comparing Cap-Hel peptides with Hel peptides, in general, the clearing behavior is not so different, although a slight difference was observed regarding the power, itself.

Hemolytic and Antimicrobial Activities. The hemolytic activity of the peptides against the human erythrocytes and their potential to inhibit the growth of different species of bacteria were investigated. Ampicillin served as a control in antimicrobial assay. Dose-response curves for the hemolytic activity of the peptides are shown in Fig. 4. Table 2 gives the MIC for a representative set of test bacteria, which includes a Gram-positive species and two Gram-negative species. The hemolytic behavior of the Cap-Hel series is the same as that of the Hel series; the increasing hydrophobicity of the peptides leads to an increasing release of hemoglobin. The ability of the Cap-Hel series is more effective than that of the Hel-series. On the other hand, all of the peptides investigated here did not show any antimicrobial activity. This suggests that neither the free nor blocked termini of peptides has any effect on the antimicrobial activity.

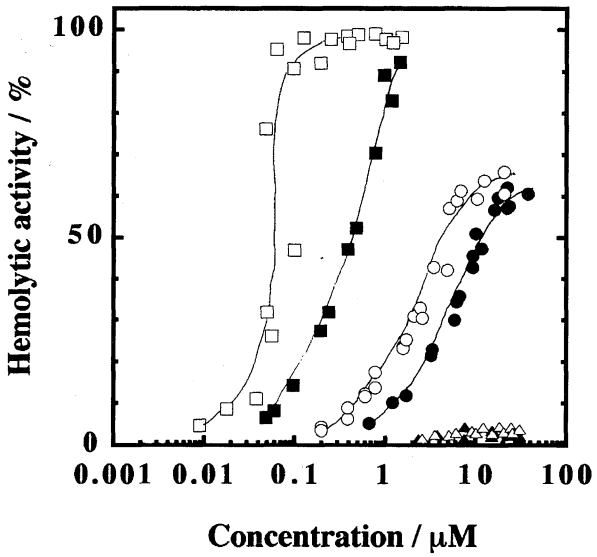


Fig. 4. Dose-response curves for hemolytic activity induced by Cap-Hel peptides and the corresponding Hel peptides. The symbols are as follows: (▲) Hel 5-13, (△) Cap-Hel 5-13, (●) Hel 9-9, (○) Cap-Hel 9-9, (■) Hel 13-5, (□) Cap-Hel 13-5.

Table 2. Antimicrobial Activity of Model Peptides

	MIC ($\mu g\,ml^{-1}$)			
	S. aureus	*E. coli*	*P. aeruginosa*	Reference
Hel 5-13	>100	>100	>100	6)
Hel 9-9	>100	>100	>100	6)
Hel 13-5	>100	>100	>100	6)
Cap-Hel 5-13	>100	>100	>100	Present work
Cap-Hel 9-9	>100	>100	>100	Present work
Cap-Hel 13-5	>100	>100	>100	Present work
Peptide 3	64	64	128	7)
Ampicillin	3.13	6.25	>100	

Discussion

In a previous study, the hydrophobic–hydrophilic balance (HHB), determined by the magnitude of the hydrophobic face and hydrophilic face of amphiphilic α-helix, was shown to affect to a significant degree the peptide–peptide and peptide–lipid interaction modes.[6] For Hel-series peptides having different hydrophobicities, but almost the same hydrophobic moments, which resulted in an ideal amphiphilic α-helical structure with systematically varied HHB, it was found that the increasing hydrophobic face area could increase not only the affinity for lipid bilayers, but also the trend of self-association. The hydrophobic moment is defined by the degree of the amphiphilic character of a helix perpendicular to its axis.[18] The present results concerning the Cap-Hel series peptides also came up to the line. In general, blocking of the both terminals by acetyl and amide groups, respectively, should lead to an increase in the hydrophobicity due to the disappearance of cationic and anionic charges. It should also lead to an increase in the helical content because of the increased number of amide groups possible to form hydrogen bonds, which may stabilize the α-helical structure, and thus may result in an increase in the hydrophobic faces.[12] As expected, the retention times in RP-HPLC for the Cap-Hel series were later, and the α-helical contents measured by CD became larger than those for the corresponding Hel series peptides, respectively.

It has been known that the interaction of amphiphilic α-helical peptides with turbid phospholipid liposomes changes the structure of the liposomes and produces a clear micellar solution of the peptides and lipids.[16] In fact, in neutral liposomes the Cap-Hel series peptides decreased the liposome turbidity with the increasing hydrophobic face, and in acidic liposomes they occasionally led to an increase or decrease in the liposome turbidity. Similar turbidity changes were also observed for the Hel series. Related to this, we previously proposed a working hypothesis for the interaction mode of Hel-series peptides with neutral and acidic liposomes.[6] According to our idea, Cap-Hel 13-5 may make neutral liposomes solubilize to a micellar solution, while it may make acidic liposomes fuse to form larger liposomes. Cap-Hel 9-9 may solubilize acidic liposomes to a micellar solution, and Cap-Hel 5-13 may make acidic liposomes turbid by their aggregation. Such an assumption was completely regenerated experimentally in the present study, as described above. This is also being supported by our preliminary experiments concerning the size determination of the lipid-peptide complex after the incubation of peptides with liposomes by means of dynamic light-scattering measurement using a NICOMP Submicron Particle Sizer Model 370 with an argon laser (data

not shown).

Hence, it is noted that the clearing power of Cap-Hel 13-5 was much weaker than that of Hel 13-5 in neutral liposomes, and was stronger in acidic liposomes. This may be correlated to a lack of cationic and/or anionic groups at both terminals. In Fig. 5, our hypothesis assumes that Cap-Hel 13-5 partially immerses the hydrophobic group in neutral or acidic lipid bilayers in a horizontal manner, as previously shown for Hel 13-5.[6] Then, in the case of neutral liposomes the peptide makes them micelles via an discoidal shape, while in the case of acidic ones the peptides immersing into liposomes lead to fusion with other liposomes via charge interaction. Cap-Hel 13-5 may immerse more easily in lipid bilayers than does Hel 13-5, because the former is larger in hydrophobicity than the latter. Thus, in neutral liposomes the lack of a charged group in Cap-Hel 13-5 may make discoidal or micellar structure destabilized because of a decrease of the hydrophilic group, resulting in a decrease in the clearing power; in acidic liposomes, however, it stabilizes the immersed state in lipid bilayers to promote the charge interaction between liposomes, resulting in fusing them to each other (Fig. 5).

The model- and erythrocyte membrane perturbation abilities of the Hel series peptides were completely in parallel to the increase of the hydrophobic region.[6] The present study also shows that the Cap-Hel series peptides cause stronger hemolysis than do the Hel-series peptides, indicating that the increasing hydrophobicity of amphiphilic peptides leads to a more effective hemolytic ability (Fig. 4). However, concerning the antimicrobial activity, Cap-Hel peptides exhibit no activity against either Gram-positive or -negative bacteria, similar to Hel peptides (Table 2). It should be noted that peptide 3, having a slightly different sequence with no Trp, but the completely same hydrophobic region as Cap-Hel 9-9 (see helical wheels of both peptides in Fig. 1), has shown a weak but distinct ability (Table 2).[7] It is unlikely that such a difference in either sequence or amino acids leads to a disruption of the amphiphilic structure. This and our results

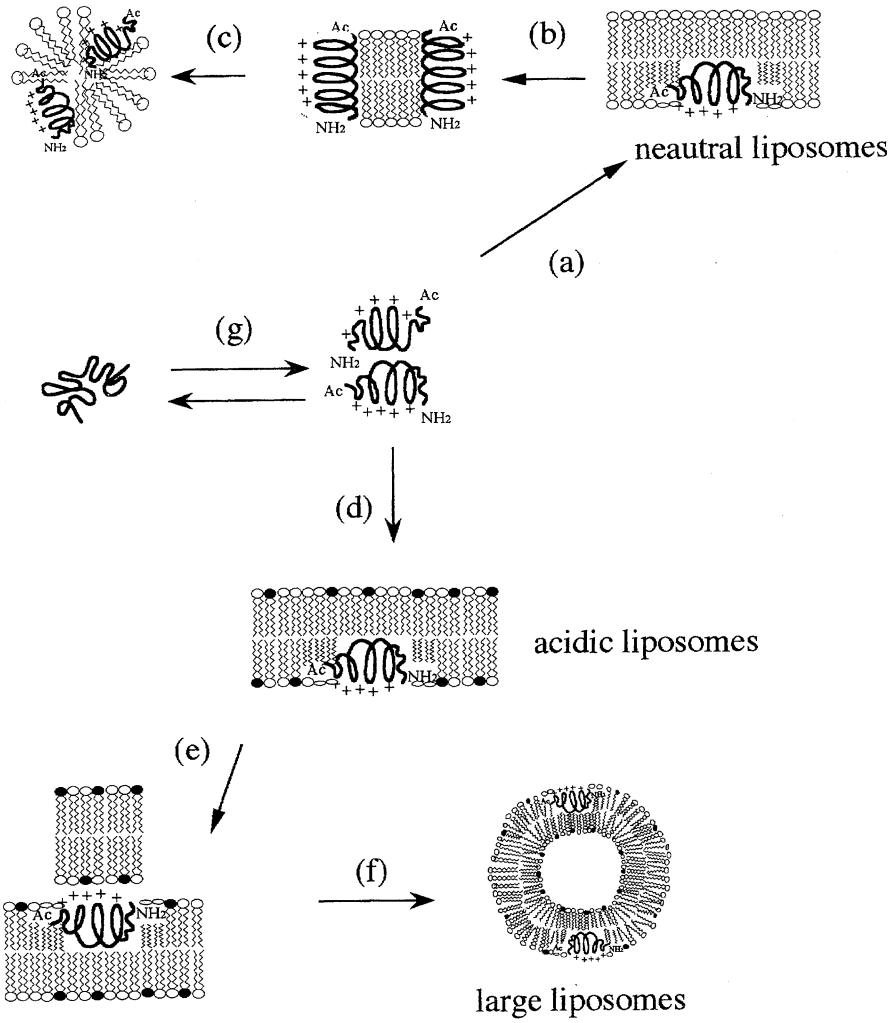


Fig. 5. Schematic representation of lipid-peptide interaction mode by Cap-Hel 13-5. Pathways (a) and (d); the peptides are immersed into lipid bilayers in horizontal manner; (b) discoid formation; (c) micelle formation; (e) the peptides immersed in lipid bilayers interact with the other lipid bilayers by charge interaction; (f) fusion to form large liposomes. The head group of acidic phospholipid is marked by black.

may suggest that a subtle difference in the hydrophobic and hydrophilic balance between Peptide 3 and Cap-Hel 9-9 may be related to the antimicrobial activity. In this connection, the antimicrobial activities of the 14-mer amphiphilic peptide Ac-LLKLLKKLLKKLKK-NH_2 and mono-Pro substitution analogs were found to be inversely correlated with their RP-HPLC retention times.[17] Melittin, which is amidated at the C-terminus, did not contribute significantly to the antimicrobial activity.[5] In contrast, cecropin with a free carboxylic C-terminal had a significantly lower activity than did that of the native amidated cecropin.[19] The recent cellar localization studies of an amphiphilic C- and N-terminal labeled peptide have shown that chemical modifications of the C- or N-terminals strongly modifying their biological activity are mainly related to variations of the binding affinities toward the targets of the peptides, and not to a modification of the localization mode of the peptides into lipid bilayers.[20] Our present results are interpreted along the same line, because acetylation of the N-terminal and amidation of the C-terminal did not affect their intrinsic antimicrobial activity in spite of a considerable change in the binding properties and hemolytic activities.

Amphiphilic antimicrobial peptides and cell lytic peptides of 13-40 residues occur in natural sources and act to perturb the membranes. A positively charged residues as well as an amphiphilic structure are also required to exhibit lytic activity. Two mechanisms have so far been proposed:[2] One is an ion-channel formation through membrane bilayers, as observed for cecropin; the other is a disruption of the ordered structure of the membrane. The accumulation of peptides in the outer half of the bilayers result in the release of membrane fragments and a simultaneous enhancement of the permeability, as observed for melittin and magainin.[21,22] The latter mechanism has been supported by a recent report that diastereomer melittin, whose two amino acids in the sequence were replaced by the corresponding D-amino acids, binds to the bacteria membrane surface in a carpet-like manner.[5] The blocking group of both termini may work effectively for the former mechanism, but not for the latter case, because the ability to form channels is favored by the large hydrophobicity for spanning lipid bilayers. Our present experimental results showed that the blocking of both terminals increased the hydrophobicity and helix-forming ability. Thus, for the channel formation mechanism needed to span lipid bilayers by taking an amphiphilic α-helical structure, protection of peptide terminal may work effectively, but for a carpet-like mechanism. In the latter case, a proper number of hydrophilic residues and hydrophobic residues in amphiphilic structure may be needed to keep and cover the peptides at the membrane surface. Peptide 3 may inhibit the growth of bacteria by means of the latter case. Unexpectedly, therefore, the capping of Hel 9-9 leads to a less effective way to inhibit the growth of bacteria.

However, the capping of both termini of Hel-series peptides led to a considerable increase in the hemolytic activity (Fig. 4). It is generally accepted that many amphiphilic compounds can alter the shape of erythrocyte from normal discoid to a echinocyte form by accumulating molecules in outer leaflet of membranes. The model peptide molecules may be accumulated in outer membranes in such a manner, resulting in their perturbation. The outer half of the erythrocyte comprises mainly neutral PC and phosphatidylethanolamine (PE), and no acidic phospholipid. This may explain why Cap-Hel 13-5 has strong hemolytic activity, in spite of its solubilization of neutral liposomes, but no solubilization of acidic liposomes, as described in the Results section. Additionally, it is said that many sialic acid molecules are located in the surface of erythrocyte membranes. Their anionic charges may allow the basic peptide to stay at the surface of the membranes. On the other hand, Cap-Hel 5-13, having many charged residues, cannot still penetrate their hydrophobic parts into an erythrocyte lipid bilayer, because of low hydrophobicity.

On amphiphilic α-helical peptides acting with biomembranes and disrupting them, it is concluded that the acetylation of N-terminal and amidation of C-terminal did not affect their intrinsic biological activity in spite of considerable changes in the lipid-binding properties and hemolytic activity; even so, only a slight change in the antimicrobial activity take place. Thus, for the *de novo* design and synthesis of peptide drugs exhibiting biological activity by acting on bio-membranes, either termini-free or protective peptides may be equally chosen if a slight difference in activity is of no concern.

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