Design and Synthesis of Amphiphilic α-Helical Model Peptides with Systematically Varied Hydrophobic−Hydrophilic Balance and Their Interaction with Lipid- and Bio-Membranes†

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ABSTRACT: Five amphiphilic α-helical peptides of 18 residues containing a hydrophobic Trp residue as a fluorescence probe were designed. The peptides were made up of hydrophobic Leu and hydrophilic Lys residues of a ratio of 13:5, 11:7, 9:9, 7:11, and 5:13 (abbreviated as Hels 13−5, 11−7, 9−9, 7−11, and 5−13, respectively). These peptides generate ideal amphiphilic α-helical structures, which have systematically varied hydrophobic−hydrophilic balance (relative amphiphilic potential) as a result of different hydrophobicities and almost the same hydrophobic moments. Their hydrophobic−hydrophilic balance was estimated both theoretically from the calculated hydrophobicity values (or the magnitude of hydrophobic faces) and experimentally from the retention times in reverse phase high-performance liquid chromatography (RP-HPLC). Circular dichroism, liposome-lytic, and Trp-fluorescent studies in buffer and in the presence of acidic and neutral liposomes clearly showed that the increasing hydrophobic face area not only increases the affinity for lipid but also increases the trend of self-association. The structure−activity relationship estimated by means of leakage ability and hemolytic activity demonstrated that the model- and bio-membrane perturbation ability is completely parallel to the magnitude of the hydrophobic face area. The lipid-binding study in guanidine hydrochloride solution showed that the peptides with a hydrophobic face larger than the hydrophilic face (Hels 13−5 and 11−7) immerse their hydrophobic regions in lipid bilayers and that the inverse ones (Hels 7−11 and 5−13) interact only between the anionic lipid head groups and cationic peptide residues on liposome surfaces. The peptide Hel 9−9, which has exactly the same hydrophobic and hydrophilic regions, was found to be at a critical boundary among these peptides in terms of (1) behavior of peptide self-aggregation in buffer solution and membrane perturbation ability, (2) transfer from bulk solution to neutral lipid bilayers, and (3) necessity of charge interaction in lipid−peptide binding.

The amphiphilic α-helical structure or β-structure has a common structural feature in cell-lytic and antimicrobial peptides that act by perturbing the barrier function of membranes (Kini & Evans, 1989; Saberwal & Nagaraji, 1994; Segrest et al., 1990). The amphiphilic α-helix is defined as an α-helical structure in which the amino acid residues are distributed in the secondary structural form of opposite polar and nonpolar faces. These naturally occurring cell-lytic and antimicrobial peptides have varying chain lengths (∼13−40 residues). Recent studies for understanding potency and specificity by showing hemolytic and antimicrobial activity using *de novo* peptides have shown a requirement for the proper chain lengths and amino acid compositions as well as the amphiphilic structure. For example, among the peptides comprised of a 3:1 ratio of apolar residues (Leu and Ala)[1] and basic ones (Lys and Arg) which varied in length from 4 to 16 residues, the highest antimicrobial activity was found for 12-mer sequence (Lee et al., 1986). A similar result has shown that the highest antimicrobial activity was obtained from the 14- and 15-mer sequences among a series of amphiphilic peptides composed of Leu and Lys (a 1:1 ratio) which varied in length from 8 to 22 residues (Blondelle & Houghten, 1992). For hemolytic activity, among a series of 12−22-mer long peptides, which were constituted of only Leu and Lys (a 2:1 ratio), the 15-, 20-, and 22-mer peptides are 5−10 times more active than melittin from bee venom, one of the highest hemolytic toxins among naturally occurring cell-lytic peptides (Cornut et al., 1994). These results suggested that proper chain length, composition (a 1−3:1 ratio), and positioning of apolar and positively charged residues are needed for hemolytic and antimicrobial activity.

The structure of lipid−peptide complexes is mainly determined by the following three interactions; the hydrophobic interaction of the apolar face of the helices with lipid, the polar interaction of the hydrophilic sides of different helices with each other, and the interaction of hydrophilic residues with aqueous solvent (McLean, 1991). Therefore relative magnitude of hydrophobic and hydrophilic faces of amphiphilic α-helical peptides should bring about the differences in hemolytic and antimicrobial activity. Relating

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[1] Abbreviations: CD, circular dichroism; egg PC, egg yolk phosphatidylcholine; egg PG, egg yolk phosphatidylglycerol; Gu·HCl, guanidine hydrochloride; RBC, red blood cell; RP-HPLC, reverse phase high-performance liquid chromatography; TFA, trifluoroacetic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane. All amino acids are of the L-configuration.

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to this, Brasseur (1991) has attempted a classification of the different lipid-associating helices by the hydrophobic and hydrophilic angles that are defined as a sector formed by hydrophobic residues and hydrophilic residues when the amphiphilic helical structure is represented by the helical wheel. If the hydrophobic angle is greater than 180°, then the helix will pack as a transmembrane pore; if the hydrophobic angles is equal to 180°, then the helix will lie parallel to the membrane surface; and if the hydrophobic angle is less than 180° the helix will solubilize the lipid bilayers by forming discoidal particles, the periphery of which is surrounded by the helices. Tytler et al. (1993) have developed Brasseur's proposal to the reciprocal wedge hypothesis that amphiphilic helices with wedge-shaped (hydrophobic angle > 180°) or inverted wedge-shaped cross-sections (hydrophobic angle < 180°) mimic the effects of wedge-shaped or inverted wedge-shaped phospholipids in lipid phases, and thus one class can neutralize the effect of the other class in lipid membranes. However, the effect of hydrophobic−hydrophilic balance (attributable to the different magnitude of apolar and polar surfaces determined from the different amino acid compositions) on membranelytic activity and biological activity has not yet been practically and systematically studied.

In the present study, we designed and synthesized five amphiphilic α-helical peptides with a set of hydrophobic−hydrophilic balances (difference in hydrophobicity) produced by varying the ratio of Leu and Lys, but with the same hydrophobic moment (the same amphiphilicity), and investigated their structure−activity relationship by means of liposome-lytic and leakage experiments and hemolytic and antimicrobial assays.

MATERIALS AND METHODS

Amphiphilic Peptides Synthesized. All peptides were synthesized by FMOC (9-fluorenylmethoxycarbonyl) chemistry starting from FMOC-(Leu or Lys)-PEG-PS (polyethylene glycol-polystyrene)-resin (0.6 g, 0.12 mmol) using a Milligen automatic peptide synthesizer model 9050 in monitoring the deprotection of FMOC group by UV-absorbance. After the cleavage from the resin by trifluoroacetic acid (22.4 mL) containing thioanisole (3.5 mL), 1,2-ethanedithiol (1.8 mL), and *m*-cresol (0.6 mL) for 2 h at room temperature, the crude peptides obtained were passed through a Sephadex G-25 gel filtration chromatography (25 mm × 130 cm). Then the peptides were purified by a preparative C18 HPLC column (20 mm × 250 mm, YMC) except for Hel 13−5 and C8 HPLC column (20 mm × 250 mm, Cosmocil) for Hel 13−5, with a gradient system of water−acetonitrile containing 0.1% TFA. The yields obtained after purification were about 30−200 mg. The purity of all peptides was confirmed by using an analytical C18 RP-HPLC column (4.6 mm × 250 mm, Cosmocil) was >98%. Amino acid analysis was performed after hydrolysis in 5.7 M HCl containing 4% thioglycolic acid and 0.6% phenol in a sealed tube at 110 °C for 24 h. Analytical data of all peptides were in good agreement with theoretical ones. Peptide concentrations in solution were determined from UV-absorbance of Trp at 280 nm in buffer ($\epsilon = 5500$) containing 6 M Gu·HCl.

Preparation of Liposome. Phospholipid [egg PC or egg PC−egg PG (3:1)] (20 mg, about 25 μmol) in chloroform (2 mL) was placed in a round-bottom flask. After evaporation of the solvent, residual film was dried under reduced pressure overnight. The lipid film was hydrated with 3 mL of Tes buffer (5 mM Tes, pH 7.4/100 mM NaCl) for CD, liposome-lytic, and lipid-titrating experiments and with a buffer (20 mM Tes, pH 7.4/150 mM NaCl) containing 70 mM calcein for leakage measurement (Matsuzaki et al., 1989). The suspensions were vortexed for 20 min. This turbid liposome solution was employed for liposome-lytic experiments. For other experiments, the solution was sonicated under ice-cooling and nitrogen flowing for 10 min (×3) by using a titanium tip sonicater. The liposomes trapping calcein were subjected to gel filtration through a Sepharose 4B column (1 cm × 20 cm) in 150 mM NaCl/20 mM Tes buffer (pH 7.4). Two-milliliter fractions were collected, and fraction number 7, which was just before the non-encapsulated dye-elution, was used for the leakage measurement. The lipid concentration of fraction 7 was about 3.8 mM. Liposome concentrations of elutes obtained by gel filtration were determined by Phospholipid Test Wako (Wako Pure Chemical Co., Osaka, Japan).

CD Spectrum Measurement. The circular dichroism spectra were recorded on a JASCO J-600 instrument with a personal computer (NEC PC-9801). Spectra were measured at 20 μM and 550 μM of peptide and lipid [egg PC liposome and egg PC−egg PG (3:1)] concentrations, respectively, except for the case of Hels 5−13 and 7−11 in egg PC:egg PG (7:1) with 10 μM and 1 mM, respectively. The α-helical contents (f_h) were calculated with the following equation (Wu et al., 1981)

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

where $[\theta]_{222}$ is experimentally observed mean residue ellipticity at 222 nm. 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 −30 000 deg cm^2/dmol, respectively (Chen et al., 1974; Chang et al., 1978).

Liposome-Lytic Experiment. Turbid liposomes prepared as described above after vortexing were diluted to a concentration of about 100 μM with the same buffer (5 mM Tes, pH 7.4/100 mM NaCl). Peptide solutions were then added to the solution to attain a given mole ratio of peptide to lipid, and the samples were incubated at 25 °C. The transmittance of sample solutions was recorded at 400 nm.

Fluorescence Study. Spectroscopic titration of peptides with liposomes was performed as reported by Surewicz and Epand (1984). For titration study by lipid, appropriate aliquots of egg PC or egg PC−egg PG (3:1) liposomes (2.0 mM) were successively added to a solution (2 mL) containing peptides (10 μM). After each addition of liposomes, the mixture was kept at 25 °C for 5 min to achieve equilibration. For denaturation studies, Gu·HCl was added to a solution of peptides and lipids to a final concentration of 6 M solution. After 5 min, the spectra were taken.

Leakage Measurement. A liposome solution (50 μL) of fraction 7 obtained as described above was added to 2 mL of 150 mM NaCl/20 mM Tes buffer (pH 7.4) in the cuvette to give a final concentration of 90 μM lipid. To the mixture was added an appropriate concentration of peptides in phosphate buffer. The leakage of calcein out of the liposome (Matsuzaki et al., 1989) was monitored by measuring fluorescence intensity at 520 nm (excitation at 490 nm) on

a JASCO FP-777 spectrofluorimeter. The cuvette was placed in the heated cuvette holder of the fluorimeter at 25 °C. For determination of 100% dye-release, 10 μL of Triton X-100 solution (10% in Tes buffer) was added to dissolve the vesicles. The percentage of dye-release caused by the peptides was evaluated by the equation

$$\text{dye-release (\%)} = 100 \times (F - F_0)/(F_t - F_0)$$

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, respectively.

Hemolytic Assay. The hemolytic activities of the peptides were determined using human red blood 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-buffered saline (PBS: 10 mM phosphate buffer, pH 7.4/150 mM NaCl) to remove plasma and buffy coat. A suspension of 0.3% hematocrit (2×10^7/mL) in PBS with or without peptide was incubated for 90 min at 25 °C. Hemolysis was expressed as hemoglobin content (absorbance at 542 nm) of the supernatant after centrifugation at 2500 rpm for 5 min. A 100% hemolysis was determined by the hemoglobin release after the addition of 0.3% Triton X-100.

Antimicrobial Activity. The minimum inhibitory concentrations (MICs) of the growth of microorganisms were determined by the standard agar dilution method using Muellar Hinton agar (Dafco), as described previously (Okonogi et al., 1986). The final bacterial suspension contains 10^6 colony-forming units (CFU)/mL.

RESULTS

Design of Amphiphilic Peptides

The peptides consist of 18 amino acid residues formed by appropriate composition of Leu and Lys residues. Lys and Leu have a strong helix-forming potential in globular proteins but quite dissimilar physical properties. On the basis of a regular α-helical structure of 3.6 residues per turn, an initial length of 18 residues was chosen so that five complete turns of an α-helix were possible. A Trp residue was introduced in the hydrophobic region of amphiphilic helical structure to monitor peptide−peptide and lipid−peptide interaction by means of fluorescence. The primary structure of the peptides and their helical wheel representation are shown in Figure 1. The sector angles increased in five steps, 40° from Hel 5−13 to Hel 13−5, starting with the hydrophobic region of 100° in Hel 5−13. The hydrophobicities and hydrophobic moments of the peptides are listed in Table 1. As seen in helical wheels, the hydrophobic region is designed to increase in parallel with increasing hydrophobicity without appreciably altering the hydrophobic moment (amphiphilicity). The experimental validity of hydrophobic−hydrophilic balance of the peptides designed was proven by their RP-HPLC retention times. To examine quantitatively the hydrophobic−hydrophilic properties, the mobility of RP-HPLC was often determined (Armstrong & Carey, 1982). For amphiphilic helical peptides, Houghten and co-workers (1987) have also shown that the secondary structures of peptides, which are induced when bound to the hydrophobic C-18 stationary phase, influence the retention times. The retention times of the present peptides (Figure 2) are

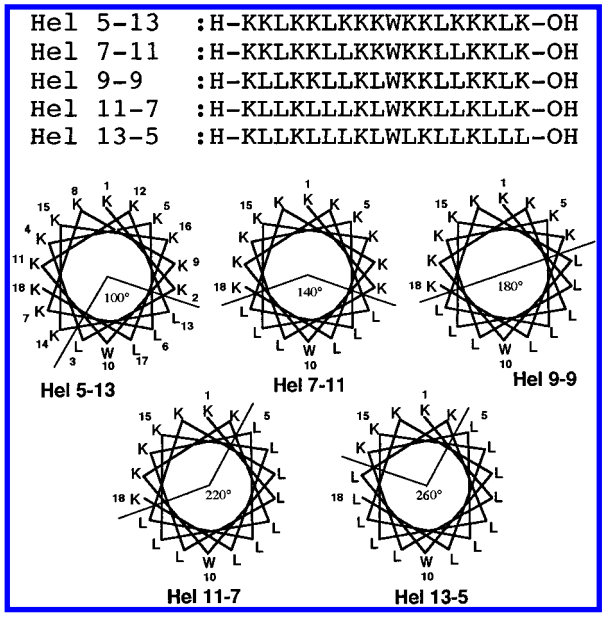

FIGURE 1: Primary structures of amphiphilic α-helical peptides and their helical wheel representations.

Table 1: Calculated Hydrophobicity, Hydrophobic Moment, and Retention Time of Model Peptides

peptide	hydrophobicity[a]	hydrophobic moment[a]	retention time (min)[b]
Hel 5−13	−0.66	0.39	16.0
Hel 7−11	−0.48	0.48	22.1
Hel 9−9	−0.29	0.51	28.5
Hel 11−7	−0.11	0.48	34.1
Hel 13−5	0.07	0.39	40.5

[a] These mean values were calculated using consensus value of hydrophobicity scale for each amino acid residue (Eisenberg, 1984). [b] This was obtained using C18 RP-HPLC at linear gradient of acetonitrile−water containing 0.1% CF_3COOH.

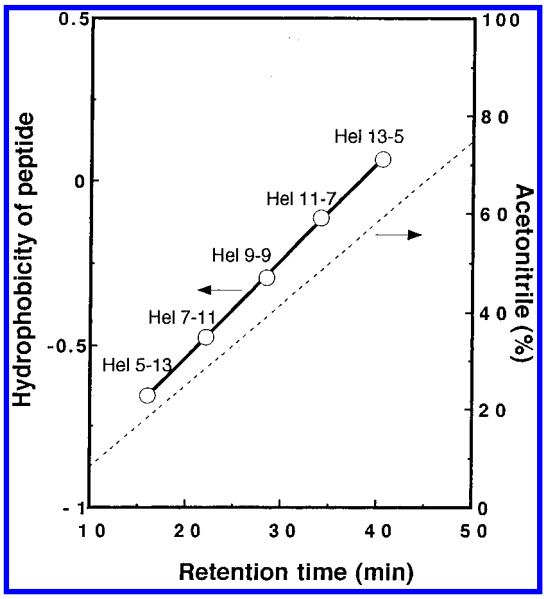

FIGURE 2: Effect of the hydrophobicity of peptides on RP-HPLC retention time. The dotted line represents percentage of acetonitrile.

completely parallel to the values of their calculated hydrophobicities (Table 1). The hydrophobic region formed by the secondary structure, which has contact with C-18 stationary phase, is the determining factor in peptide retention time. All the peptides took α-helical structure in TFE, which

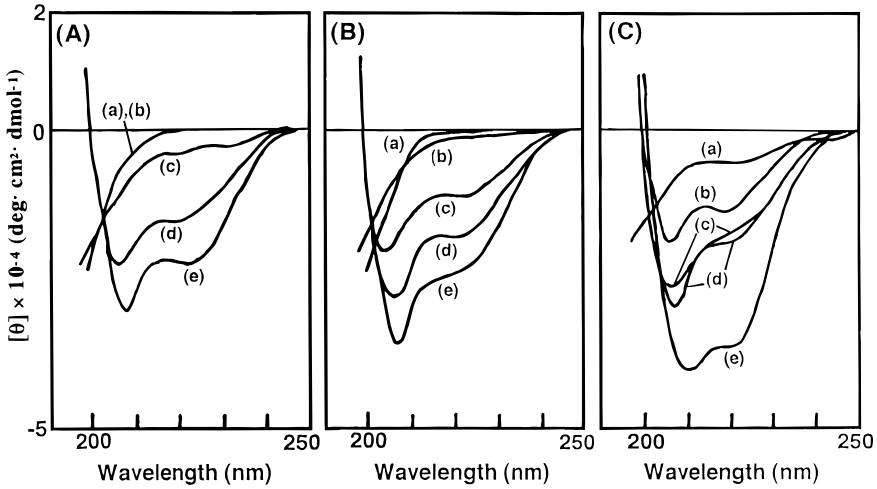

FIGURE 3: Circular dichroism spectra of amphiphilic model peptides in 5 mM Tes buffer containing 100 mM NaCl at pH 7.4 (A), in the presence of egg PC liposome (B) and egg PC−egg PG (3:1) liposome (C). Spectra were measured at 20 μM and 550 μM of peptides and lipid concentrations, respectively, except for the case of Hels 5−13 and 7−11 in egg PC:egg PG (7:1), which were at 10 μM and 1 mM, respectively. Hels 5−13, 7−11, 9−9, 11−7, and 13−5 are symbolized as (a), (b), (c), (d), and (e), respectively.

Table 2: α-Helical Contents of Amphiphilic Peptides in Various Media[a]

peptide	80% TFE	buffer	egg PC[b]	egg PC:egg PG
Hel 5−13	0.34	0	0	0.12[c]
Hel 7−11	0.35	0	0	0.34[c]
Hel 9−9	0.41	0.03	0.22	0.45[d]
Hel 11−7	0.47	0.42	0.50	0.50[d]
Hel 13−5	0.45	0.68	0.75	1.0[d]

[a] CD spectra were measured at 25 °C. Peptide concentrations were kept at 20 μM except for the case of b, the case of which was 10 μM. [b] Molar ratio of peptide and lipid was 1:25. [c] Ratio of egg PC:egg PG was 7:1. Molar ratio of peptides and lipids was 1:100. [d] Ratio of egg PC:egg PG was 3:1. Molar ratio of peptide and lipid was 1:25.

is an amphiphilic solvent as described later. This means that the hydrophobicity values calculated can be used as a measure for the hydrophobic−hydrophilic balance, when peptides take an α-helical structure, in such amphiphilic surroundings similar to that in TFE.

CD Study

Secondary structure of the peptides was characterized by CD measurement in various media (Figure 3 and Table 2). In 80% trifluoroethanol, all peptides showed the double-minimum CD curves at around 208 and 222 nm, characteristic of α-helical structure. Their helical contents are about 35−45%, indicating that the peptides designed have an inherent helicity in a hydrophobic membrane-like milieu (aqueous trifluoroethanol). In buffer solution (5 mM Tes, pH 7.4/100 mM NaCl) with a peptide concentration of 20 μM, the lower hydrophobicity peptides (Hel 5−13, 7−11, and 9−9) take random structure while Hels 11−7 and 13−5 take α-helical structure, the contents of which increase with increasing hydrophobicity. It is expected that an amphiphilic secondary structure would form readily "micellar structure", that is, small oligomers formed with a well-defined number of monomers, where the lipophilic domains are strongly interacting. Therefore, these CD experimental results suggest that Hels 11−7 and 13−5 with the larger apolar face (>180°), self-associate with their hydrophobic faces formed by adopting the amphiphilic α-helical structure, but the former three peptides with smaller apolar face (<180°), are present as a monomeric state with random structure under the above peptides and buffer concentrations. These oligomeric or monomeric behavior of the peptides in buffer solution will be also supported from fluorescence study of Trp residue in the peptides as described later.

Similar CD curves as those in aqueous solution were observed in the presence of neutral liposomes, except that the helical content of Hel 9−9 was considerably higher than that in buffer. The α-helical contents of Hels 9−9, 11−7, and 13−5 increase with their increasing hydrophobic faces. But Hels 5−13 and 7−11 still remain random structures, suggesting no interaction with neutral lipid. In the presence of acidic liposomes, the helical contents of Hels 9−9 and 13−5 became much higher than those in buffer or in the presence of neutral liposomes, although the helical content of Hel 11−7 did not vary in either media. No CD data of Hels 5−13 and 7−11 were obtained for liposomes containing a high ratio of acidic phospholipid ([acidic lipid]/[neutral lipid] = 1:3) because of vigorous turbidity of the liposome solutions after addition of the peptides possibly due to aggregation or fusion. When measured in liposomes containing a low ratio of acidic phospholipid ([acidic lipid]/[neutral lipid] = 1:7), Hels 5−13 and 7−11 also took α-helical structure, indicating that charge interaction between lipid and peptide plays a definite role for the helix formation and/or liposome aggregation or fusion.

Liposome-Lytic Activity of Peptides

Interaction of the peptides with neutral and acidic lipid bilayers was monitored by change in the turbidity of liposomes with time after incubation of the peptides at 25 °C (Figure 4A and B). In the clearing of the egg PC liposomes, the most effective peptides in the series were Hels 11−7 and 13−5, but it should be noted that the clearing power of Hel 11−7 is slightly higher than that of Hel 13−5 in several attempts (see Figure 4A). This is interesting because the magnitude of hydrophobicity is usually parallel to the solubilizing power of lipid. On the other hand, Hels 9−9, 7−11, and 5−13 were poor at lipid clearing. This is compatible with the results of the CD experiments that these peptides weakly or scarcely took α-helical structure in the presence of neutral liposomes.

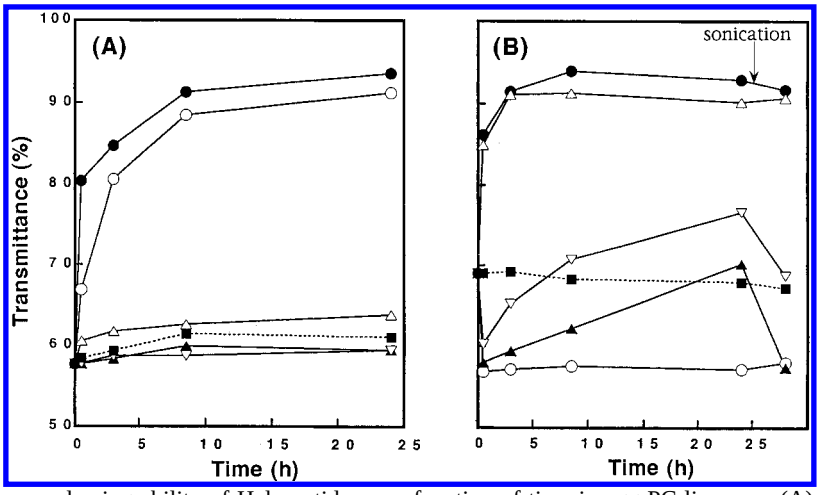

FIGURE 4: The turbid liposome-clearing ability of Hel-peptides as a function of time in egg PC liposome (A) and egg PC−egg PG (3:1) liposome (B). The peptides were incubated in turbid liposomes prepared by vortexing of hydrated lipid films in buffer at pH 7.4. In (B), the incubated solutions were sonicated weakly with a bath type sonicater at 25 h (arrow). The transmittances of sample solutions were recorded at 400 nm. Those of turbid liposome themselves (■) as standard solutions are 55% for egg PC liposomes and 70% for egg PC−egg PG liposomes. Peptide concentrations are 20 μM for Hels 13−5 (○), 11−7 (●), and Hel 9−9 (△) and 100 μM for 7−11 (▲) and 5−13 (▽). Lipid concentration is 100 μM.

Table 3: Maximum Emission Wavelengths of Amphiphilic Peptides in Various Media[a]

			egg PC				egg PC:egg PG (3:1)			
			[P]:[L]		[P]:[L]		[P]:[L]		[P]:[L]	
peptide	buffer[b]	Gu·HCl[c]	(1:25)[d]	Gu·HCl[c]	(1:100)[d]	Gu·HCl[c]	(1:25)[d]	Gu·HCl[c]	(1:100)[d]	Gu·HCl[c]
Hel 5−13	357	−	357	−	357	−	349	358	336	357
Hel 7−11	357	−	357	−	356	−	334	356	329	355
Hel 9−9	355	−	344	356	328	356	326	355	327	354
Hel 11−7	342	355	328	332	328	330	326	335	326	331
Hel 13−5	332	352	323	320	323	321	320	325	320	322

[a] Fluorescence spectra were measured at 25 °C and excitation was at 280 nm. Peptide concentration was 10 μM. [b] 5 mM Tes, 100 mM NaCl, at pH 7.4. [c] Gu·HCl concentration was 6 M. [d] The values in parentheses represent molar ratio of peptide and lipid.

In acidic liposomes composed of egg PC−egg PG (3:1), Hels 11−7 and 9−9 were very effective in clearing the liposome turbidity (see Figure 4B). Contrary to this, addition of Hels 13−5, 7−11, and 5−13 to turbid liposomes resulted in a rapid and drastic increase in turbidity within 30 min. While the turbidity for Hel 13−5 did not change after about 30 min, those for Hels 7−11 and 5−13 again start to decrease with time after about 30 min. When a weak sonication was tried under the condition where the turbidity of the control solution did not change, it was found that the turbidity of Hels 7−11 and 5−13 again dropped. This suggests that Hels 13−5, 7−11, and 5−13 increase the turbidity of liposomes but with a different mechanism; Hel 13−5 does it by fusion of liposomes, and Hels 7−11 and 5−13 does it by aggregation. In the former case, an assembly becomes larger by fusion within 30 min (turbidity increases), and it cannot dissociate with the weak sonication (no change of turbidity). On the other hand, in the latter case, an assembly becomes larger with time by aggregation (turbidity increases), and then it dissociates easily with the weak sonication (turbidity decreases). Here it should be noted again that Hel 13−5 makes neutral liposomes clear strongly; in contrast, when mixed with the acidic liposomes, it makes them turbid. The clearing power of Hel 9−9 is strong in acidic liposomes but not in neutral liposomes.

Fluorescent Study

The fluorescence change of a single Trp residue in the peptides was examined in various media (Table 3). In buffer, the emission maximum wavelengths of Trp residues in Hels 5−13, 7−11, and 9−9 were around 355 nm, indicating the solvent exposure of Trp and of Hels 11−7 and 13−5 were around 340 and 330 nm, respectively, indicating hydrophobic environment of Trp by self-aggregation of the peptides. Addition of egg PC liposomes results in a large blue shift in the emission maximum for Hels 9−9, 11−7, and 13−5, indicating that the peptides bind to lipid, but no blue shift was observed for Hels 5−13 and 7−11. As for binding to lipid, a distinct difference was observed for binding between Hel 9−9 and Hel 11−7 when titrating the peptides with egg PC liposomes (Figure 5A and B, respectively). For Hel 9−9, the fluorescence intensity of maximum peak decreased gradually at a concentration ratio below [L]:[P] = 30:1 and then increased slightly to reaching maximum equilibrium at [L]:[P] = 60:1. For Hel 11−7, the intensity of maximum emission peak increased with the increasing lipid concentration and reached equilibrium at [L]:[P] = 2:1. These results indicate that Hel 11−7 binds to lipids smoothly and strongly, and Hel 9−9 binds to them in at least two stages.

In the presence of acidic liposomes, the emission maximum of Hel 5−13 shifted to blue wavelengths slightly, and that of Hel 7−11 moderately, indicating that neither Trps are present at a high hydrophobic environment. These blue shifts increased with the increasing lipid composition for peptides. The maxima of Hels 13−5, 11−7, and 9−9 shifted strongly to blue wavelengths, indicating that each Trp is present in a hydrophobic core in lipid bilayers.

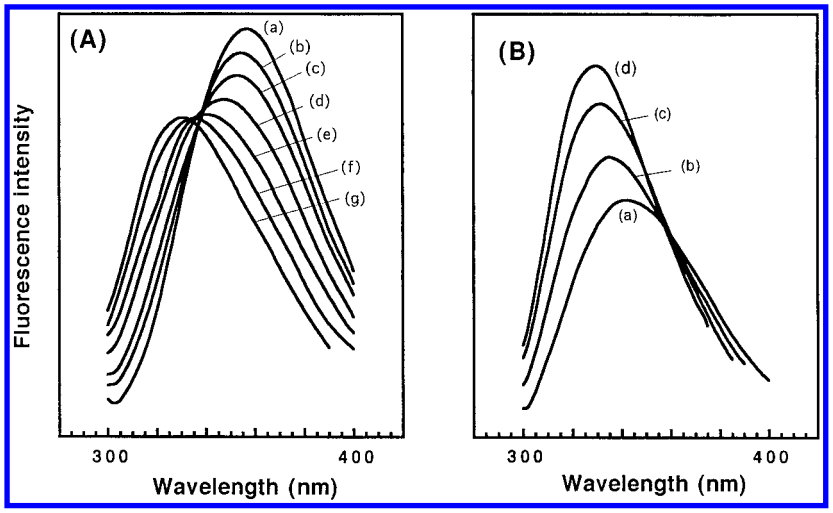

FIGURE 5: Trp fluorescence spectra of Hel 9−9 (A) and Hel 11−7 (B), when titrated with increasing concentrations of egg PC liposomes in buffer solution at pH 7.4. Peptide concentrations are 10 μM. Lipid concentrations are (A) 0 (a), 60 (b), 120 (c), 230 (d), 335 (e), 463 (f), and 652 μM (g) and (B) 0 (a), 6 (b), 12 (c), and 18 μM (d).

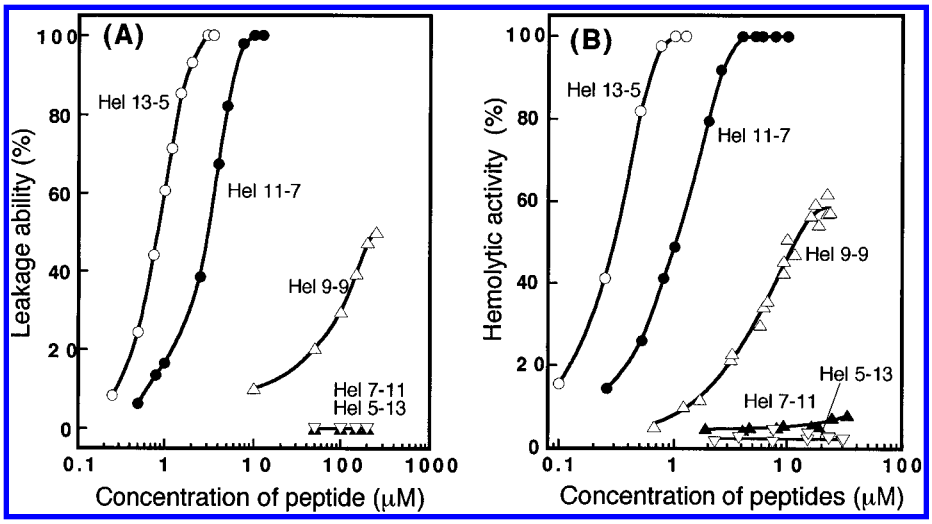

FIGURE 6: Dose−response curves for leakage ability in egg PC liposomes (A) and hemolytic activity (B) induced by the peptides. Lipid concentration was 80−90 μM (A), and number of red blood cells is 2×10^7/mL (B).

To attain more precise information of binding of peptides to liposomes, a Gu·HCl solution was added to the peptide-incubated liposome solution (Table 3). It is apparent that amphiphilic α-helical peptides are immersed into lipid bilayer only by taking α-helical structure. Thus, the peptides which penetrate hydrophobic parts into lipid bilayers should not change their fluorescent behavior even in buffer solution containing such a denaturant as Gu·HCl. The maximum wavelength value (λ_{max}) of Trp fluorescence was examined by adding Gu·HCl; the resultant λ_{max} values for Hels 13−5 and 11−7 were scarcely changed by Gu·HCl in the presence of neutral and acidic liposomes, indicating the strong penetration of the hydrophobic parts of the peptides into lipid bilayers. On the other hand, those of Hels 9−9, 7−11, and 5−13 are at about 355 nm even in the presence of acidic liposomes, indicating that the peptides do not immerse their hydrophobic parts into lipid bilayers. It is reasonable to consider that when Gu·HCl does not exist in the liposome solution, Hel 9−9 is distributed in an equilibrium state between the phospholipid bilayers with α-helical structure and the bulk solution with random structure, but the disruption of the α-helical structure by addition of Gu·HCl to the solution leads all the peptides to stay in the bulk solution.

Model- and Bio-Membrane Perturbation Studies

To evaluate the interaction of the peptides with model membranes and biomembrane, the ability of the peptides to leak dye from neutral liposome and hemoglobin from human blood cells was measured. Their dose−response curves are shown in Figure 6. Incubation of calcein-entrapped egg PC liposomes with model peptides resulted in leakage of the content in a dose-dependent manner for Hels 13−5 and 11−7 but not for Hels 7−11 and 5−13. Hel 9−9 did not induce complete dye-release even at high concentrations (50 μM), indicating that it has a moderate ability between higher and lower hydrophobicity peptides. Thus leakage ability of the peptides is parallel to their hydrophobicity; the larger the hydrophobicity, the stronger the leakage ability.

The same results as the dye-release experiment were obtained for dose−response curves for the hemolytic assay as shown in Figure 6B. Hemolysis decreased with decreasing hydrophobic region, but Hel 5−13 did not show any activity. These results indicate that the abilities of model- and bio-membrane perturbations can be estimated by hydrophobic−hydrophilic balance of amphiphilic peptides.

None of the peptides showed antimicrobial activity against either Gram-positive or -negative bacteria and fungi (mini-

mum inhibitory concentrations, >100 μg/mL) such as *Staphylococcus aureus* FDA 209 P, *Bacillus subtilis* PCI 219, *Escherichia coli* NHIJ JC-2, and *Pseudomonas aeruginosa* U-31.

DISCUSSION

A concept on hydrophobic−hydrophilic balance [as for this terminology, refer to Armstrong and Carey (1982)] has been introduced to explain micelle formation, solubilization, and adsorption behavior of surfactants, particularly bile salts in the bodies of vertebrates which are related to their physiological membranolytic functions (Sugihara et al., 1995; Fahey et al., 1995). In the present study, the concept was applied on membrane-associating peptides to explain their physicochemical and biological properties. Relating to this, there are few studies that address the relationship between the hydrophobicity and membrane-associating properties of amphiphilic peptides: (1) a hydrophobic plot related to the hydrophobic moment and hydrophobicity of the segment (Eisenberg, 1984); (2) a classification of the lipid-associating helices based on their molecular hydrophobic potential (Brasseur, 1991); and (3) the reciprocal wedge hypothesis by analogy to the reciprocal effect of phospholipid shapes on membrane structures (Tytler et al., 1993). In such studies, the lipid binding properties of the peptides largely depend on their hydrophobic surfaces that are estimated by the mean hydrophobic and hydrophilic angles of sector that represent the isopotential line around the helix. In the present study, hydrophobic−hydrophilic balance of peptides was estimated from almost the same and relatively high hydrophobic moments ($< \mu H = 0.35-0.50$) and the different hydrophobicities brought about by various hydrophobic angles (260°, 220°, 180°, 140°, and 100°). To determine experimentally the quantitative hydrophobic−hydrophilic balance of the amphiphilic substance, RP-HPLC has been often employed (Armstrong & Carey, 1982; Houghten & Degraw, 1987). The present RP-HPLC study showed that when peptides can take α-helical structure, the relation of the peptides−retention times with hydrophobicities calculated was completely parallel, namely, that the hydrophobic−hydrophilic balance of the peptides was measured by the hydrophobicities or hydrophobic angles.

The peptide−lipid and peptide−peptide interactions of amphiphilic helical peptides are expected to lead to an increase in the α-helicity, a decrease in the solvent exposure of tryptophan, and occasionally an increase or decrease of turbidity of liposomes. The present conformational, liposome-lytic, and Trp-fluorescent studies in buffer and in the presence of liposomes clearly showed that the increasing hydrophobic face enhances not only the affinity for lipid but also the affinity of the peptides for itself (assembling tendency).

Hel 13−5, with the largest hydrophobic face, revealed the highest helical content and the largest blue shift of Trp fluorescence in both buffer and lipid media, meaning that the ability of self-association and binding-to-lipid is the strongest among the model peptides. However, the clearing ability of Hel 13−5 for turbid liposomes is slightly less than that of Hel 11−7 in neutral liposomes and surprisingly Hel 13−5 accompanied an increase of turbidity in acidic liposomes. It is considered that interaction of amphiphilic α-helical peptides with turbid phospholipid liposomes disrupts the structure of the liposomes to produce a clear micellar solution of peptides and lipid. This process consists of (1) binding of the peptides to the lipid bilayers, (2) insertion and self-association of the peptides in lipid bilayers, and (3) reconstruction of the liposomes into disks. It may be interpreted as follows: too strong self-association of Hel 13−5 weakens its dissociation even in neutral liposomes, resulting in inhibiting transfer to the third step of phospholipid solubilization. In the case of acidic liposomes, the charged interaction between peptide and lipid head group as well as the strong hydrophobic binding may keep the peptide on the membrane surface in the horizontal manner as described later (Figure 7d), resulting in fusion with other liposomes.

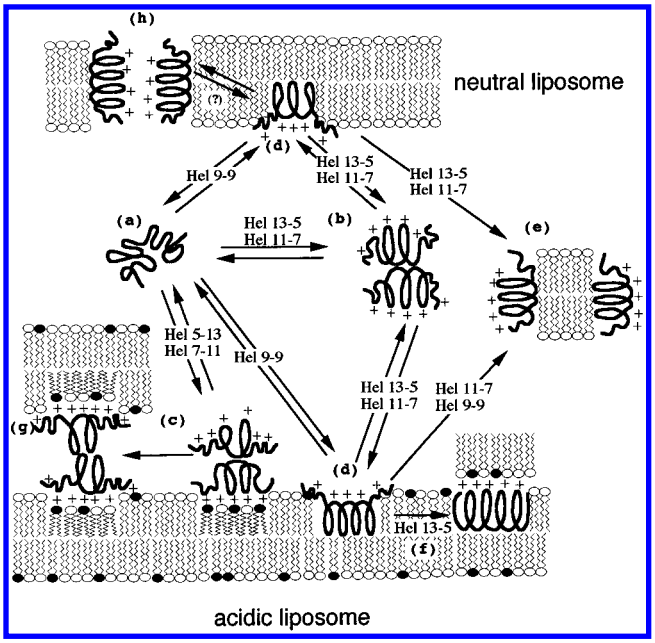

FIGURE 7: Schematic representations of lipid−peptide interaction mode by Hel peptides: (a) a random form in bulk solution (Hels 9−9, 7−11, and 5−13); (b) aggregated form in bulk solution (Hels 13−5 and 11−7); (c) charge interaction of peptide with lipid bilayers in acidic liposomes (Hels 5−13 and 7−11); (d) partially immersed peptide horizontally into lipid bilayers (Hels 9−9 and/or Hels 13−5 and 11−7); (e) micellar formation in neutral liposomes (Hels 13−5 and 11−7) and in acidic liposomes (Hels 11−7 and 9−9); (f) aggregation probably to fuse acidic liposomes (Hel 13−5) (g) aggregation of acidic liposomes (Hels 7−11 and 5−13); and (h) a hole that possibly leaks dye (Hels 13−5, 11−7, and 9−9). The head group of acidic liposome is marked by black.

Hel 9−9 lies in the critical boundary region, the boundary line of which is drawn in terms of the behavior of peptide self-aggregation in buffer solution, the possibility of transferring of the peptides from bulk solution to neutral lipid bilayers and the extent of needing a help of charge interaction on peptide−lipid interaction. It is interesting that Hel 11−7 expected to have intermediate behavior between Hel 9−9 and Hel 13−5 showed a solubilizing ability being almost equal to Hel 13−5 for neutral lipids but as for acidic lipid equal to Hel 9−9. It is noted that the α-helical contents of Hel 11−7 are about 50% in all media and the value is also about the same as that of Hel 9−9 in acidic liposomes (Table 2). This means that Hel 11−7 can bind to lipid bilayers without help of charge interaction. Moderate association and dissociation in lipid bilayers as compared with those of Hel 13−5 may make Hel 11−7 transfer to the third step, resulting in solubilizing lipid membranes. Hel 9−9 can also solubilize

lipids with the help of charge interaction between the lipid head group and cationic peptides.

From titration study of Hels 11−7 and 9−9 with neutral phospholipid liposomes, distinct differences are also found in interaction mode of both peptides with lipid bilayers. That is, Hel 11−7 showed a gradual increase in the maximum wavelength as well as the intensity of fluorescence, suggesting an insertion of the aggregated peptide in buffer solution into more hydrophobic lipid bilayers. On the other hand, Hel 9−9 interacted in a two-stage manner, decreasing and then increasing of fluorescence intensity accompanied by shifting to shorter wavelength with increasing lipid concentration. Such fluorescence change may reflect a gradual translation from the aggregated state in buffer to the penetrated state in lipid bilayers.

Next, looking at Hels 7−11 and 5−13, these took α-helical structures and led to a blue shift of Trp fluorescence only in the presence of acidic lipid bilayers. Both peptides increased the turbidity of the liposomes and then the turbidity decreases with time. This might mean that the driving force of α-helical structures induced by lipid bilayers comes from charge interaction between anionic lipid head groups and cationic Lys residues. Such interaction seems similar to α-helical structures induced by polylysine in the presence of acidic liposomes (Hammes & Shullery, 1970; Fukushima et al., 1989). In this occasion, the hydrophobic region of the amphiphilic structure will be shallowly immersed into the membrane and/or associate with other molecules. The latter case is more likely, because liposome solutions become vigorously turbid when the peptides are added to liposomes containing acidic phospholipid at high ratio of acidic/neutral phospholipid (3:1); this is probably due to aggregation or fusion (Figure 7c and g).

The lipid−peptide interaction mode described above is also found in a fluorescent study in the presence of Gu·HCl. When the peptides were added to the neutral and acidic liposomes under the condition where all peptides were present as a random structure in buffer solution containing Gu·HCl, the Trp residues of Hels 13−5 and 11−7 were still sealed from the hydrophilic environment, meaning that the hydrophobic parts of peptides were immersed into lipid bilayers. On the other hand, the Trp residues of Hels 7−11 and 5−13 are in the solvent exposure. The Trp of Hel 9−9 is also in the solvent exposure in acidic liposomes as well as neutral liposomes in the presence of Gu·HCl, suggesting that in normal buffer solution, the Hel 9−9 distributes at equilibrium between the solution and the lipid bilayers as described in the Results.

The model- and RBC-membrane perturbation abilities are completely parallel to the increase of hydrophobic region. It should be mentioned that since abilities of both hemoglobin from blood cells and dye from model liposomes have almost the same potency (Figure 6). This finding excludes possibilities such as perturbation of membrane proteins or specific interaction with lipids, e.g., glycolipid and cholesterol. Hel 13−5 has the highest hemolytic activity (LD_{50} = 0.5 μM), comparable with melittin. Increased hydrophobicity could be expected to result in a deeper penetration into a membrane, leading to a strong hemolytic activity. Relating to this, Dufourcq and co-workers (1994) reported that N-dansylated amphiphilic 18-mer peptide [composed of Lys (5) and Leu (13), having the same hydrophobic angle as Hel 13−5] has a considerable hemolytic activity (LD_{50} = 4.7 μM; this value is 5−6 times less than Hel 13−5). An 18-mer peptide composed of Lys (9) and Leu (9), Ac-LKLLKKLLKKLKKLLKKL-NH_2 having the same hydrophobic angle as Hel 9−9, exhibited hemolytic activity at 100 μg/mL (about 40 μM) that was only 10−15% of lysis obtained according to the treatment of erythrocytes with Triton X (Blondelle & Houghten, 1992). This value is almost comparable to Hel 9−9. It is concluded that the peptides having hydrophobic angle less than 180° can exhibit none or drastic reduction of hemolytic activity.

As for antimicrobial activity, however, the peptides exhibited nothing against either Gram-positive or -negative bacteria. The interaction mode for the model membrane is the same as that for the erythrocyte membrane but seems different for bacterial cells. It is interesting that a weak but distinct activity (60−120 μM) was observed for Ac-LKLLKKLLKKLKKLLKKL-NH_2 having a different sequence with no Trp but the same hydrophobic angle as Hel 9−9 (Blondelle & Houghten, 1992). When the N- and C-terminals of Hel 9−9 are acetylated and amidated, respectively, the peptide (Ac-Hel 9−9-NH_2) has no antimicrobial activity (data not shown). It is unlikely that such a difference in either sequence or amino acid results in disruption of amphiphilic structure or loss in helical content. It was found that peptides composed of 17 amino acid residues (named modelin), containing highly hydrophobic (mainly due to Trp or Phe) and hydrophilic residues (due to Lys), showed a considerable antimicrobial and significant hemolytic activity. Meanwhile the modelin-related peptides in which Trp or Phe of modelin was replaced with Leu exhibited a drastic decrease in hemolytic and only a slight decrease in antimicrobial activity (Bessalle et al., 1993). These results suggest that a subtle difference in hydrophobic and hydrophilic balance affects such antimicrobial activities. In addition, there is a report that a peptide composed of 20 amino acids derived from pig myeloid DNA with the same hydrophobic angle of 100° as Hel 5−13, exhibits a potent antimicrobial activity (Storici et al., 1994). It seems that critical chain length for the peptides having hydrophobic angle of about 100° may be present at 18−20-mer residues to show antimicrobial activity.

In general, it has been estimated that chain length of 20 amino acid residues in an α-helical structure is required to span a lipid bilayer. We previously reported that on two amphiphilic α-helical peptides composed of 24 and 20 amino acid residues with hydrophobic angle similar to Hel 13−5, the former showed a channel activity with weak antimicrobial activity but the latter showed an increase of elastic current instead of no channel formation and no antimicrobial activity (Agawa et al., 1991). A 14-mer seminal plasmine-related peptide (Sitaram & Nagaraji, 1990), 14-mer mastoparan B (Park et al., 1995), 17-mer modelin (Bessalle et al., 1993) and 15-mer peptides composed of Lys and Leu (a ratio of 1:2) (Cornut, 1994) having about 180°−260° of hydrophobic angles, exhibit strong or moderate hemolytic activity. These results suggest that the mechanism of hemolysis of Hels 13−5, 11−7, and 9−9 are not due to channel formation.

Tytler et al. (1993) have proposed a reciprocal wedge hypothesis in which amphiphilic helices with wedge-shaped (hydrophobic angle > 180°) or inverted wedge-shaped cross-sections (hydrophobic angle < 180°) can neutralize action of the other class on lipid membranes. However, addition of Hel 11−7 to erythrocyte membrane pre-incubated by Hel

7−11 did not inhibit release of hemoglobin (data not shown). As Hel 11−7 and Hel 7−11 can be taken as wedge- and inverted wedge-shape, the present peptide−lipid interaction mode might be explained by a mechanism other than reciprocal wedge hypothesis.

In the present study we have shown that the hydrophobicity of the hydrophobic face as well as a large value of hydrophobic moment determines to a significant degree the peptide−peptide and peptide−lipid interaction mode. On the basis of the present study, we propose here the working hypothesis for the mode of interaction of Hel-series peptides with lipid bilayers as shown in Figure 7: (a) a random form in bulk solution (Hels 9−9, 7−11, and 5−13); (b) an aggregated form in bulk solution (Hels 13−5 and 11−7): (c) charge interaction of peptide with lipid bilayers in acidic liposomes (Hels 5−13 and 7−11); (d) partially immersed peptides form horizontally into lipid bilayers (Hel 9−9, and/or Hels 13−5 and 11−7); (e) micellar formation in neutral liposomes (Hels 13−5 and 11−7) and acidic liposomes (Hels 11−7 and 9−9); (f) aggregation of acidic liposomes probably to fuse (Hel 13−5); (g) aggregation of acidic liposomes (Hels 7−11 and 5−13); (h) a hole (?) that possibly leaks dye (Hels 13−5, 11−7, and 9−9).

In conclusion, the results of the present study of the relationship of structure and lytic- and bio-activity indicated that hydrophobic−hydrophilic balance, namely, the proportions of positively charged and hydrophobic moieties, specifies the lipid−peptide interaction mode. This may facilitate not only understanding of the mechanism of action of surface-seeking peptides and proteins but also the design of potent novel biological drugs.

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