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Effect of amphipathic peptides with different α-helical contents on liposome-fusion

Sannamu Lee [1], Reiko Aoki [1], Osamu Oishi [1], Haruhiko Aoyagi [1] and Nobuyuki Yamasaki [2]

[1] *Laboratory of Biochemistry, Faculty of Science, Kyushu University, Higashi-ku, Fukuoka (Japan) and* [2] *Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University, Higashi-ku, Fukuoka (Japan)*

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The peptide-induced fusion of neutral and acidic liposomes was studied in relation to the amphiphilicities evaluated by α-helical contents of peptides by means of a carboxyfluorescein leakage assay, light scattering, a membrane intermixing assay and electron microscopy. An amphipathic mother peptide, Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$ (4_3), and its derivatives, [Pro^6]4_3 (1), [Pro^{2,6}]4_3 (2), and [Pro^{2,6,10}]4_3 (3), which have very similar hydrophobic moments, caused a leakage of contents from small unilamellar vesicles composed of egg yolk phosphatidylcholine and egg yolk phosphatidic acid (3:1). The abilities of the peptides to induce the fusion of the acidic liposomes increased with increasing α-helical content: in acidic liposomes the helical contents were in the order of $4_3 > 1 > 2 > 3$ (Lee et al. (1989) Chem. Lett., 599–602). Electron microscopic data showed that 1 caused a transformation of the small unilamellar vesicles (20–50 nm in diameter) to large ones (100–300 nm). Based on the fact that these peptides have very similar hydrophobic moments despite of decreasing in the mean residue hydrophobicities to some extent, it was concluded that the abilities of the peptides to induce the fusion of liposomes depend on the extent of amphiphilic conformation evaluated by α-helical contents of the peptides in the presence of liposomes. For neutral liposomes of egg yolk phosphatidylcholine, all the proline-containing peptides showed no fusogenic ability but weak leakage abilities, suggesting that the charge interaction between the basic peptides and acidic phospholipid is an important factor to induce the perturbation and fusion of the bilayer.

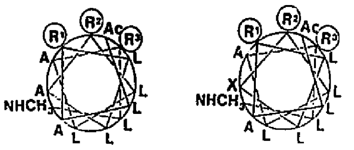
Introduction

The peptide–lipid interaction has been extensively studied to understand the general rules of the membrane fusion [1]. To understand the molecular details of this process, the interaction of peptides or proteins with lipids has been studied on model systems such as phospholipid bilayers. An amphipathic peptide is ideally suited to interact with amphipathic surface such as cell membranes or liposomes. In the preceding paper [2], we reported that basic amphipathic α-helical peptides caused the destabilization and fusion of acidic and neutral liposomes, and their fusogenic abilities were closely associated with their hydrophobic moments when the peptides took an α-helical structure. Recently, Kono et al. reported that an amphipathic and basic sequential peptide, poly(Lys-Aib-Leu-Aib), induced a fusion of DPPC liposomes in an alkaline solution below the phase transition temperature of the membrane [3]. A similar result has also been obtained for an acidic amphipathic α-helical peptide in acidic pH regions in the presence of neutral liposomes [4]. These findings have indicated that a pH-dependent conformational change is essential for triggering the interaction of the peptide with a lipid bilayer to induce the membrane fusion and that the fusogenic ability of amphipathic α-helical peptides against artificial membranes is correlated to the α-helix formation of the peptides, regardless their acidities and basicities. How-

Abbreviations: egg PC, egg yolk phosphatidylcholine; egg PA, egg yolk phosphatidic acid; N-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazo-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Correspondence: S. Lee, Laboratory of Biochemistry, Faculty of Science, Kyushu University 33, Higashi-ku, Fukuoka 812, Japan.

1 5 10	
Ac-Leu-Ala-Arg-Leu-Leu-Ala-Arg-Leu-Leu-Ala-Arg-Leu-NHCH$_3$	4_3
Ac- -Pro- -NHCH$_3$	1
Ac- -Pro- -Pro- -NHCH$_3$	2
Ac- -Pro- -Pro- -Pro- -NHCH$_3$	3



4_3 1

Fig. 1. Primary structures of the basic model peptides containing proline and their α-helical wheels (X = Pro).

ever, a relationship between the extent of α-helical conformation of amphiphilic peptides and the fusogenic ability has not been studied completely.

In previous report, we presented evidence that an α-helical peptide, Ac-(L-Leu-L-Ala-L-Arg-L-Leu)$_3$-NHCH$_3$ (4_3), showed various biological activities owing to a perturbation of biomembranes [5] and was an effective fusogen for liposomes [2]. This peptide shows amphiphilicity by taking an α-helical structure with one lateral hydrophobic side and the other hydrophilic side (Fig. 1). Since proline is a typical helix-breaker amino acid, the substitution of Ala to Pro in the sequence is considered to cause the decrease in the α-helical content of the peptide, and consequently a reduction of the extent of amphiphilic conformation. We previously synthesized three analogs of 4_3 which contained one to three prolines; [Pro^6]4_3 (1) [Pro^{2,6}]4_3 (2) and [Pro^{2,6,10}]4_3 (3) (Fig. 1). Previous CD data showed that the α-helix-forming potential of the peptides in the presence of acidic liposomes is gradually reduced with an increase in the Pro residue in the peptides. The antimicrobial activity of the peptides was also reduced with the same order, indicating that the effect of the peptide on biological activity was correlated to the ability to adopt α-helical structure [6].

The membrane-related peptides and proteins have been characterized by the value of α-helical hydrophobic moment and the mean residue hydrophobicity [7,10]. Peptide 4_3 and the analogs have very similar hydrophobic moments when they take fully an α-helical structure. The hydrohobicities of the peptides gradually decreased to some extent, but not very different, with increasing proline residue. Therefore, these peptides having the same hydrophobic moments and similar hydrophobicities, but various α-helical contents in the presence of acidic liposomes are a favorable model for studying the quantitative relation between the amphiphilic structure and fusogenic abilities of peptides.

In the present work, the fusogenic abilities of the peptides with the different α-helical contents are studied with regard to their conformations in neutral and acidic liposomes.

Materials and Methods

Egg PC and egg PA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). N-NBD-PE and N-Rh-PE were purchased from Avanti Polar Lipids Inc. (Pelham, AL, USA). Carboxyfluorescein from Eastman Kodak Co. (Rochester, NY, USA) was purified by recrystallization from ethanol. All other reagents were of an analytical grade.

The peptides were prepared by solution methods as reported previously [5,6] and their purities were confirmed by thin-layer chromatography, paper electrophoresis, amino acid and elemental analyses. The peptide contents were calculated from the result of elemental analysis as follows: $4_3 \cdot 3AcOH \cdot 6H_2O$; $1 \cdot 4AcOH \cdot 6H_2O$; $2 \cdot 5AcOH \cdot H_2O$; $3 \cdot 5AcOH \cdot 6H_2O$. Peptides were dissolved in 5 mM Hepes buffer (pH 7.4) at a desired concentration whilst being stirred over a period of an hour. The solution was stocked in a refrigerator.

Small unilamellar liposomes were prepared by sonication using a Tomy Seiko Ultrasonic disrupter Model UR-200P, followed by gel-filtration as reported previously [2,5]. The buffer solution used throughout this work was 5 mM Hepes (pH 7.4) containing 100 mM NaCl. All assays and incubations were performed at 22°C. Fluorescence spectra were recorded on a JASCO FP-550A spectrofluorophotometer equipped with a thermostatted cell holder.

Leakage of vesicle contents was determined with carboxyfluorescein as reported previously [2]. The fluorescence intensity was measured at 3 min after the addition of the peptides to the liposomes. Complete release of carboxyfluorescein was obtained by the addition of Triton X-100. Turbidity measurements for assay of vesicle aggregation and size increase were performed in the JASCO spectrofluorometer with both excitation and emission monochrometers set at 545 nm at 25°C. The light-scattering intensity was measured at 10 min after the incubation of peptide with liposomes as reported previously [2]. The membrane intermixing was determined by measuring the change in the fluorescence intensity owing to the fluorescence energy transfer between the labeled probes NBD-PE and Rh-PE [9]. Two kinds of small unilamellar vesicles containing NBD-PE and N-Rh-PE (each 2 mol%) were prepared by sonication, followed by gel filtration. Equimolar aliquots of them were mixed at 25°C and diluted with the buffer solution to make the final concentration of the liposomes to be 70 μM. The peptides were added to the mixed liposomes and the decrease in NBD fluorescence was recorded continuously at an excitation wavelength of 450 nm and an emission wave-

length of 530 nm. The membrane intermixing ability of the peptide was expressed as a percentage of the fluorescence intensity. The fluorescence intensity of the mixed solution of both liposomes before the addition of the peptides was taken as 100%.

Samples for electron microscopy were prepared as previously described [2] and measured with a JEM-100C electron microscope. Liposomes were prepared to 10-times higher concentration (about 700 μM) than in other experiments to facilitate electronmicroscopic image. The peptide was incubated at the concentration of 150 μM for about 1 min with liposomes before negative-staining.

Results

Amphiphilicity of peptides

The amphiphilicity of a segment in polypeptide chains is often evaluated by the hydrophobic moment [7]. The four model peptides used here (see Fig. 1) have high and similar hydrophobic moments of about 0.50 kcal/residue when they take an α-helical structure [7]. The mean residue hydrophobicities of 4_3 and **1**–**3** are −0.12, −0.15, −0.17 and −0.20 kcal/residue, respectively [7]. All peptides therefore belong to the cluster of 'surface-associating' peptides according to Pownall et al. [10] and can be considered to bind to lipid with almost same manner.

As previously described [6], 4_3 has a slightly α-helical structure in the buffer solution and a highly α-helical one in neutral and acidic liposomes. All proline-containing analogs of 4_3 have a random structure in the aqueous solution. In neutral liposomes, **2** and **3** take a random structure and **1** takes a somewhat ordered structure. In acidic liposomes, all peptides tested showed α-helical CD bands and the band intensity decreased in order of $4_3 > 1 > 2 > 3$. The α-helical contents were calculated by the method of Chen et al. [8] on the basis of previous CD data [6]. The percentages of α-helix of 4_3 and **1**–**3** are 76, 43, 30 and 15% in acidic liposomes, respectively. These results indicate that the proline-containing peptides weakly or scarcely bind to neutral liposomes and favorably interact with acidic liposomes and the extent of the amphiphilicities of the four peptides in acidic liposomes can be expressed in terms of the α-helical content of the peptides.

Peptide-induced dye leakage from liposomes

The leakage of encapsulated carboxyfluorescein has been used for examining a change of the permeability of lipid bilayers [11]. As shown in Fig. 2, it is evident that the leakage abilities of the peptides decreased with decrease in their α-helical contents. A distinct difference in the leakage abilities of the peptides was observed in neutral liposomes which interact weakly or scarcely with peptide as found in the CD data. Peptide **1** had a fairly high leakage ability but its efficiency was lower than that observed for 4_3. Furthermore, **2** had a slight leakage ability, and **3** failed to cause the leakage of the dye from liposomes. Since **1** formed a somewhat helix-like ordered structure while **2** and **3** took no ordered structure in neutral liposomes [6], such a difference in the leakage ability can be interpreted as being due to the difference of the binding affinities according to the differerence in the conformations of the peptides in liposomes. In acidic liposomes, 4_3, **1** and **2** released the dye strongly and any noticeable difference in the leakage abilities was not observed among these three peptides at the concentration of 0.1–1 μM. Peptide **3** also showed a fairly high leakage ability. It is certain that the charge interaction between the cation of basic peptides and the head groups of the acidic liposome is more essential to release the dye than the hydrophobic interaction between amphipathic peptides and lipids.

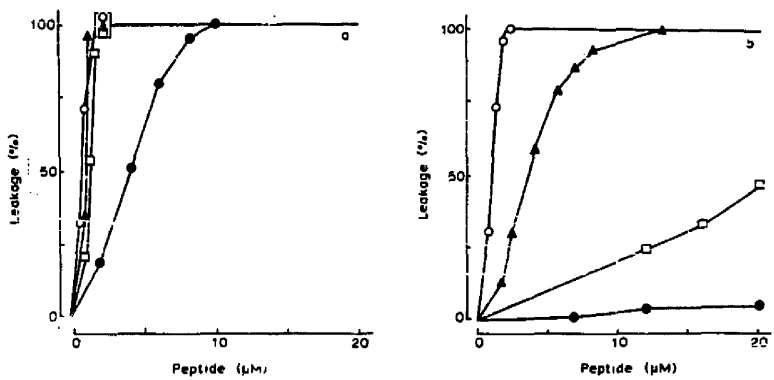


Fig. 2. Release of encapsulated carboxyfluorescein from liposomes as a function of peptide concentration. Egg PC/egg PA (3:1) (a) and egg PC (b) in the presence of 4_3 (○), **1** (▲) **2** (□) and **3** (●).

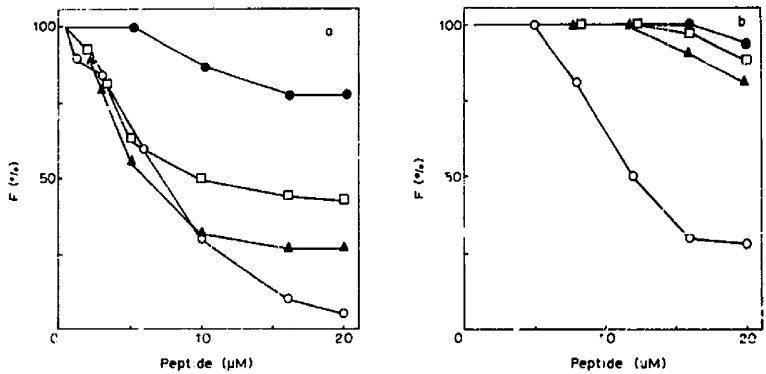


Fig. 3. Plots of membrane-mixing of egg PC/egg PA (3:1) (a) and egg PC (b) liposomes induced by the peptides as a function of peptide concentration. Data were collected at 10 min after the incubation of peptides with liposomes. 4_3, ○; **1**, ▲; **2**, □, and **3**, ●.

Intermixing assay

Fig. 3 shows the degree of the intermixing of liposome membranes as a function of peptide concentration for egg PC and egg PC/egg PA (3:1) containing N-NBD-PE and N-Rh-PE. In acidic liposomes, 4_3, **1** and **2** induced the intermixing at a concentration of 1 μM and a difference in the ability to induce intermixing was observed at a peptide concentration higher than 10 μM. Peptide **3** induced the membrane-intermixing at a concentration of 5 μM. The magnitude of intermixing decreased with decreasing helical content at a peptide concentration higher than 10 μM. These results indicate that the content of amphiphilicity estimated by the α-helix-forming ability is significant to execute the fusion of liposomes. In neutral liposomes, 4_3 which can take an α-helical structure in neutral liposomes showed a fairly high ability to induce intermixing, while **1** and **2** showed only slight abilities and no intermixing was observed for **3**. As **1**–**3** have no amphipathic structure in neutral liposomes, it is likely that the fusogenic abilities of these peptides are due to the slight difference among their hydrophobicities.

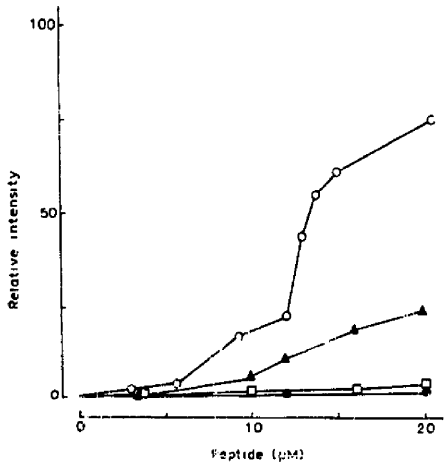


Fig. 4. Light-scattering profiles of egg PC/egg PA (3:1) in the presence of peptides. 4_3, ○; **1**, ▲; **2**, □, and **3**, ●.

Light scattering

The peptide-induced aggregation of liposomes was monitored by light scattering (Fig. 4). Among the peptides tested, 4_3 induced aggregation of acidic liposomes composed of egg PC and egg PA (3:1) most effectively, and its efficiency was about 3-times higher than that of **1**. On the contrary, peptides **2** and **3** induced the aggregation of the acidic liposomes less effectively than **1** at a concentration of 20 μM. That is clear that the aggregation efficiencies of the peptides are also correlated to the α-helical contents of the peptides in acidic liposomes. Any decrease in light scattering intensities of the peptides was not observed even at concentrations higher than 20 μM (data not shown), suggesting that the peptides did not disrupt the phospholipid bilayer to form a micellar or discoidal structure [12].

Electron microscopy

Electron microscopic data indicated that **1** induced the increase in the mass of acidic liposomes. After 1 min incubation, the diameter of the acidic liposomes consisting of egg PC and egg PA (3:1) increased from 20–50 nm to 100–300 nm (Fig. 5). The average size was slightly smaller as compared to that obtained for the liposomes enlarged by 4_3 [2]. Although **2** and **3** also caused aggregation or fusion of the liposomes, the size of the liposomes was much smaller than that induced by **1** (data not shown).

Discussion

In the preceding paper [2], we reported that the amphiphilicity of the peptides due to the formation of

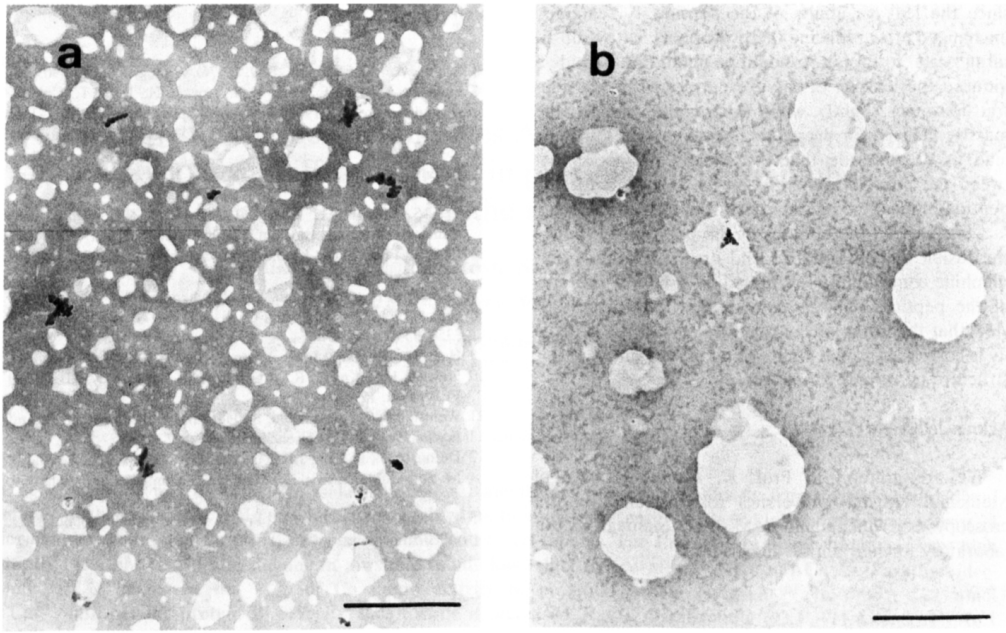


Fig. 5. Electron micrographs of liposomes. Unilamellar vesicles consisting of egg PC/egg PA (3:1) (a) were incubated with **1** for about 1 min (b). Micrographs were obtained by the negative-stain method. The bar represents 200 nm.

an α-helical structure was important for inducing the fusion of liposomes, and their fusogenic abilities were correlated to hydrophobic moments of the peptides. All the results in the present study showed that the proline-containing peptides have abilities to induce the fusion of small acidic unilamellar liposomes and their abilities depend on the α-helical contents of the peptides in the liposomes.

The values of hydrophobicities (−2.0 to −1.0 kcal/residue) and hydrophobic moments (about 0.50 kcal/residue) of these peptides fall in a 'surface associating' peptide having a low membrane mean residue hydrophobicity (−2.0 to 1.0 kcal/residue) and a high helical hydrophobic moment (0.5 to 1.25 kcal/residue) [10]. This implies that the peptides would bind to lipid bilayers in similar manner under a certain condition. In fact, the proline-containing peptide took a random structure in buffer and neutral liposomes and an α-helical structure in acidic liposomes. Since the α-helical structure was induced in the presence of acidic liposome, the binding affinity of these peptides to acidic lipid might be evaluated by their α-helical-forming potentials.

In acidic liposomes, the α-helical contents of $\mathbf{4_3}$, **1**, **2** and **3** decreased in this order. Furthermore, the abilities of the peptides to induce aggregation and fusion of liposomes as examined by light scattering and lipid mixing techniques were also parallel to the helical contents. Since their α-helical forming-potentials are correlated to the extent of their amphiphilicities, these findings indicate that the abilities of the peptides to induce the fusion of liposomes depend on the extents of amphiphilicities.

The content mixing technique used to estimate a fusion ability could not be adopted for these peptides because of too rapid initial burst of the leakage to monitor under the conditions employed [13]. However, electron microscopic data suggested that the increase in the size of liposomes induced by the peptides was not due to the exchange of the lipid molecules between liposomes but due to the fusion of liposomes (Fig. 5).

As previously reported, $\mathbf{4_3}$ induced a massive leakage [2]. It is noteworthy that all the Pro-containing derivatives of $\mathbf{4_3}$ induce a massive leakage similarly to $\mathbf{4_3}$ in acidic liposomes, even though the amphiphilicities of the peptides decrease, owing to the decrease in the α-helical contents. These findings suggest that the amphiphilicity of the peptide is not dominant for inducing the massive leakage. There is a possibility that the ability of the peptide to induce the massive leakage is dependent on the hydrophobicity of the peptide. In this case, however, this possibility can be negligible

since the leakage ability of the peptide 4_3 remained unchanged after reducing the hydrophobicity by substituting Ala(s) to Pro(s). It should be noted that peptide 3 induced the leakage of the dye entrapped in the neutral liposomes slightly, while this peptide induced the massive leakage for the acidic liposomes. These results lead us to conclude that the charge interaction between the peptide and liposome is important for the peptide-induced membrane perturbation and fusion.

Conclusively, the abilities of the peptides to induce the fusion of liposomes depend on the extent of amphiphilic conformation evaluated by α-helical contents of the peptides. This is consistent with the previous view that the effect of the peptides on biological activity is correlated to the ability of taking the amphipathic structure by α-helix formation [6].

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