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Conformational studies of amphipathic α-helical peptides containing an amino acid with a long alkyl chain and their anchoring to lipid bilayer liposomes

Tamaki Kato [1], Sannamu Lee [1], Shin Ono [1], Yukio Agawa [1], Haruhiko Aoyagi [1], Motonori Ohno [1] and Norikazu Nishino [2]

[1] *Laboratory of Biochemistry, Faculty of Science, Kyushu University, Higashi-ku, Fukuoka (Japan) and* [2] *Department of Applied Chemistry, Kyushu Institute of Technology, Tobata-ku, Kitakyushu (Japan)*

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In order to investigate the conformation and orientation of lipid-bound peptides and proteins in the lipid bilayer, basic amphipathic α-helical peptides with a long alkyl chain, palmitoyl-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$ (P-4$_3$) and Ac-Leu-Ala-Arg-Leu-Trp-Amy-Arg-Leu-Leu-Ala-Arg-Leu-NHCH$_3$ (Amy-4$_3$, Amy; α-aminomyristic acid) were designed and synthesized. The conformational features and spectroscopic behavior in a buffer solution and in neutral and acidic liposomes were studied by CD, dye-leakage, and fluorescence measurements. The CD data indicated that P-4$_3$ took an α-helical structure in aqueous solution and in neutral and acidic liposomes. On the other hand, Amy-4$_3$ took a β-structure in aqueous solution and an α-helical structure in neutral and acidic liposomes. The conformational change of Amy-4$_3$ was confirmed by fluorescence study on lipid titration of the peptide. The dye-leakage experiment showed that both peptides interacted with acidic liposomes to perturb them, but less effectively than Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$ (4$_3$) which has no long alkyl group. Based on these results, a discussion is made concerning the conformation and orientation of peptides in aqueous solution and in the lipid bilayer.

Introduction

A number of physiologically important proteins are known to have a covalently linked fatty acid which is often essential for exhibiting their biological function and activity (for reviews, see Refs. 1–4). The protein-linked fatty acid may be responsible not only for the anchoring of proteins as seen in the case of some membrane proteins, but also for maintaining the conformation of the proteins or for affecting the lipid bilayer. Two types of protein-linked fatty acid are present, one having thioester- or ester-type linkage to cysteine, serine or threonine residue, the other having amide-type linkage to the α-amino group of proteins. Interestingly, both types are not found simultaneously on the same polypeptide chain. Although it has been speculated that such highly hydrophobic modification helps with the membrane association of proteins, the effects of these highly hydrophobic lipid groups on the conformation of peptides and proteins have scarcely been studied [5].

To study the conformation and anchoring to the biomembrane of peptides with a long alkyl chain group, we designed two model peptides. One contains a fatty acyl group at the N-terminus and the other has an unusual amino acid residue with a long alkyl group in the middle of the peptide chain (see Fig. 1). An amphipathic α-helical peptide, Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$ (4$_3$), was selected as the mother peptide, because it was well-defined in the conformational aspects in the presence of the lipid bilayer and showed various

Abbreviations: Amy, α-aminomyristic acid; DPPC, dipalmitoyl-DL-α-phosphatidylcholine; egg PC, egg yolk phosphatidylcholine; egg PG, egg yolk phosphatidylglycerol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDC, 1-ethyl-(3,3′-dimethyl-aminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole; Pac, phenacyl; TFA, trifluoroacetic acid. All amino acid symbols denote L-configuration.

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

interesting biological activities due to its amphipathic nature [6–8]. In Amy-4_3, Ala^6 and Leu^5 in 4_3 are substituted by Amy (α-aminomyristic acid) and Trp, respectively, while P-4_3 is an N-palmitoylated peptide. In the present work, we studied the conformation of peptides in both an aqueous solution and in liposomes and discuss the effect of long alkyl groups on conformational change of the peptide backbone and on anchoring to the lipid bilayer.

Materials and Methods

Materials

Egg PC and egg PG were purchased from Sigma Chemical Co. Carboxyfluorescein obtained from Eastman Kodak Co. was further purified by recrystallization from ethanol. All other reagents were of analytical grade.

Synthesis of peptides

Peptides 4_3 and P-4_3 were prepared as previously reported [6,11]. The synthetic route for Amy-4_3 is shown in Fig. 2. The racemic Amy was prepared as reported by Kimura [9] and optically active L-Amy was obtained by resolution with *Aspergillus genus* aminoacylase (Tokyo Kasei) according to the literature [10]. Short fragment Boc-Trp-Amy-Arg(Tos)-Leu-OPac (I) was prepared stepwise from the C-terminus. The Boc-tetrapeptide acid (II) obtained by the treatment of I with Zn-powder in 90% acetic acid was condensed with tetrapeptide methylamide (III) to produce Boc-octapeptide methylamide (IV). The protected dodecapeptide (VII) was prepared by fragment condensation of tetrapeptide (V) and octapeptide methylamide (VI). The peptide obtained by treatment of the fully protected peptide (VIII) with HF, was applied on a Sephadex G-15 column (1.8 × 100 cm) and eluted with 30% AcOH solution. The crude Amy-4_3 obtained by lyophilization of the fraction containing the peptide, was purified by reverse-phase HPLC (TSK ODS-120T, 0.46 × 25 cm) using a 2-propanol gradient in the aqueous phase of 0.1% TFA. Data of amino acid analysis after hydrolysis in 6 M HCl revealed Ala 2.2, Leu 4.7 and Arg 3.0. Amy was not eluted under the conditions of the standard amino acid analysis using a Hitachi Model 835 High Speed Amino Acid Analyzer.

Spectroscopic experiments

All peptides were dissolved in 5 mM Hepes buffer (pH 7.4) at a desired concentration whilst being stirred over a period of 2 h. No precipitation was observed in the solution for a few days. Phospholipid concentration was determined by an assay using the phospholipid-test Wako reagent purchased from Wako Pure Chemical Industries (Osaka) and was expressed in terms of phosphorus concentration. CD spectra were recorded on a JASCO J-600 spectropolarimeter. All measurements were performed in 5 mM Hepes buffer (pH 7.4). Small vesicles (about 50 nm in diameter) were prepared as previously described [6], i.e., lipid film obtained by the evaporation of a chloroform solution of phospholipids (approx. 20 mg) was hydrated in 5 mM Hepes buffer (pH 7.4) by repeated voltex-mixing at room temperature. The suspension was sonicated for 30 min using a Tomy Seiko ultrasonic disrupter model UR-200P, in order to obtain a mixture of uni- and multilamellar liposomes at 23°C.

For measurements of CD spectra, the peptides were dissolved at a concentration of 10 μM in 5 mM Hepes buffer (pH 7.4) containing 0.9 mM egg PC or egg PC-egg PG (3 : 1) liposome solution. The solution was equilibrated for 10 min at 22°C before being measured. All measurements were performed at 22°C and the data were expressed in terms of mean residue ellipticities. To eliminate any scattering due to liposomes, each CD spectrum of liposomes was subtracted from that of the peptides measured in the presence of liposomes. A cloud of liposomes due to the addition of the peptides was not observed in the experimental conditions. The peptide conformation was evaluated by the three structures of α-helix, β-structure, and random coil according to the Greenfield and Fasman method [12].

Fluorescence spectra were recorded on a JASCO FP-550A spectrofluorophotometer at 22°C. Titration of the peptides with lipids was examined to obtain the information of peptide orientation in lipids, because the blue shift reflects the incorporation of a Trp residue into the lipid bilayer. A solution (1 ml) of Amy-4_3 at a concentration of 20 μM was added to the appropriately diluted liposome solution (1 ml) and mixed for 10 min in order for it to become equilibrated. Fluorescence intensities were corrected for blank measurements from suspension of the same concentration of liposomes in the buffer. The tryptophan fluorescence of the solution was recorded with excitation at 250 nm in order to remove any effect of the Raman scattering beam on the tryptophan fluorescence region caused by water. The leakage experiments were examined by monitoring the

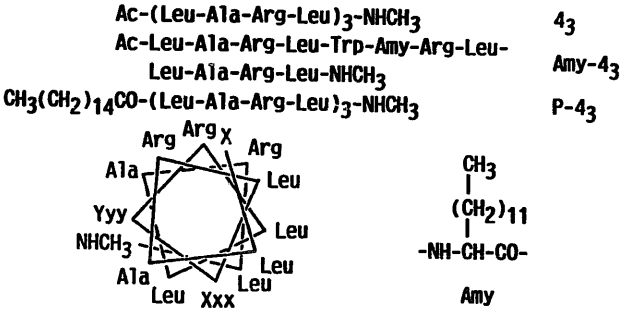

Fig. 1. Structure of model amphipathic α-helical peptides with a long alkyl chain and Amy and α-helical wheel of 4_3 (X, Ac; Xxx, Leu; Yyy, Ala), Amy-4_3 (X, Ac; Xxx, Trp; Yyy, Amy) and P-4_3 (X, CH_3-$(CH_2)_{14}$-CO-; Xxx, Leu; Yyy, Ala).

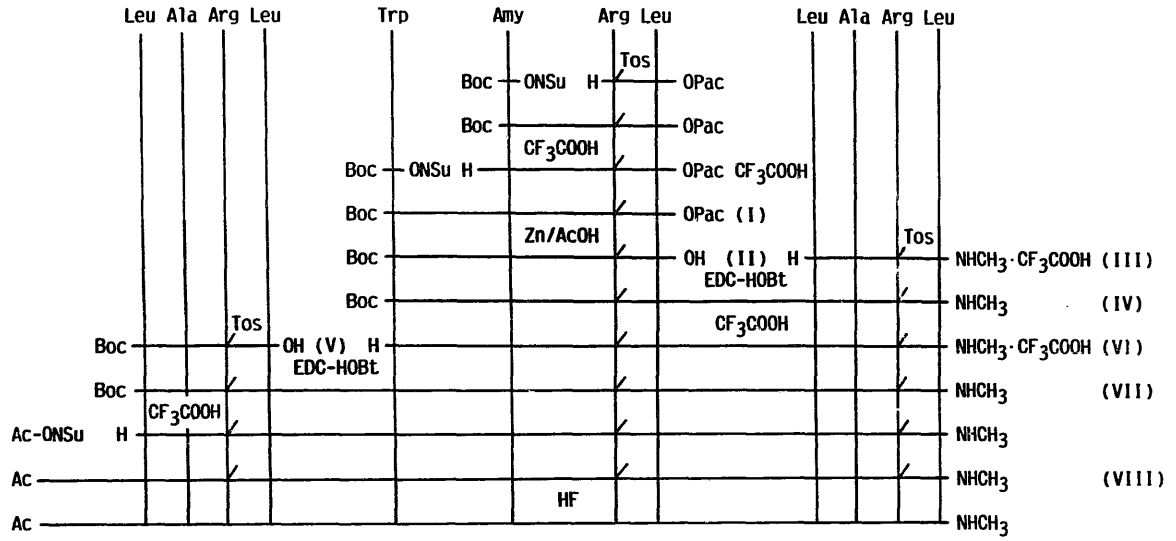

Fig. 2. Synthetic route for Amy-4_3.

release of 5(6)-carboxyfluorescein trapped in egg PC and egg PG (3 : 1) liposomes by fluorescence at 515 nm with excitation at 470 nm as described previously [6,7]. Liposomes containing 100 mM 5(6)-carboxyfluorescein were prepared by sonication as described above. After being passed through a Sepharose 4B column to remove non-entrapped carboxyfluorescein, the liposomes (2 ml, about 70 μM) were incubated at an appropriate concentration of peptides, and peptide concentration-dependent data were collected at 3 min after incubation.

Results

Design and synthesis of peptides

Peptide 4_3, selected as the mother peptide, is basic and shows amphipathic behavior when it takes an α-helical structure. The Amy and Trp residues in Amy-4_3 were introduced to retain a lipophilic region similar to 4_3, when Amy-4_3 forms an α-helical structure (Fig. 1). The unusual amino acid, Amy, was selected due to its relatively easy synthesis (Fig. 2) and its adequate hydrophobicity when compared with other fatty acids. This amino acid can also be considered as an analog of side-chain acylated cysteine or serine. P-4_3 was used as a model peptide with a long alkyl chain at the N-terminus.

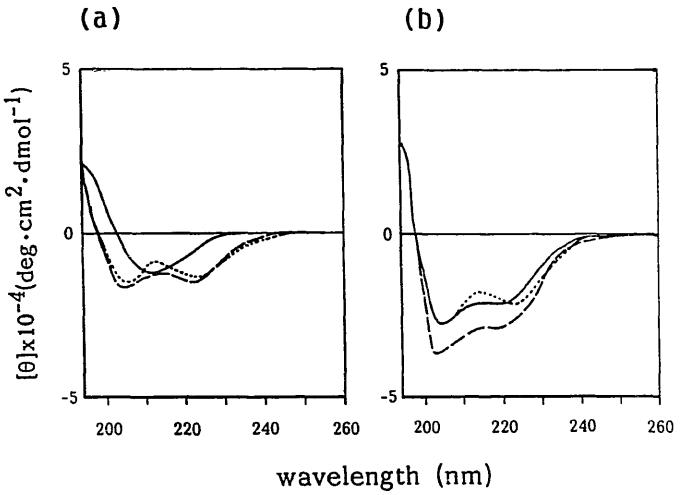

Fig. 3. CD spectra of peptides, Amy-4_3 (a) and P-4_3 (b). The spectra were measured in 5 mM Hepes buffer (pH 7.4) (———) in the presence of 0.9 mM egg PC (·····) and egg PC-egg PG (3:1) (– – –) liposomes. Peptide concentration was 10–15 μM.

CD study

Fig. 3 shows the CD spectra of P-4_3 and Amy-4_3 in the presence and absence of neutral and acidic liposomes. As shown in Fig. 3a, Amy-4_3 showed a maxi-

TABLE I

Major secondary structures of peptides Amy-4_3, P-4_3 and 4_3 (%)
α, α-helix; β, β-structure; r, random.

	Hepes buffer (pH 7.4)			Egg PC			Egg PC/egg PG (3:1)		
	α	β	r	α	β	r	α	β	r
Amy-4_3	5	60	35	40	–	60	50	20	30
P-4_3	60	–	40	60	–	40	90	–	10
4_3	–	–	100	70	–	30	75	–	25

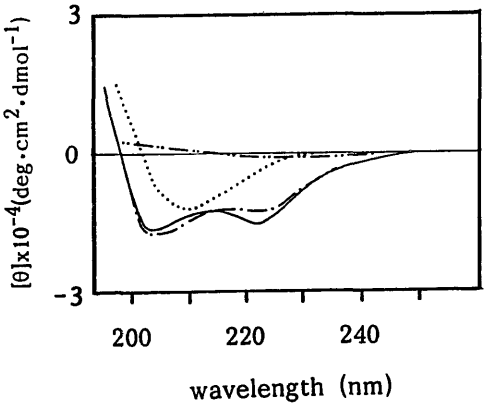

Fig. 4. CD spectra of Amy-4_3 in the presence of egg PC-egg PG (3:1) liposomes as a function of lipid concentration. Peptide concentration was 10 μM. Lipid/peptide: peptide only (·····), 3 (–··–), 20 (–·–), 80 (———).

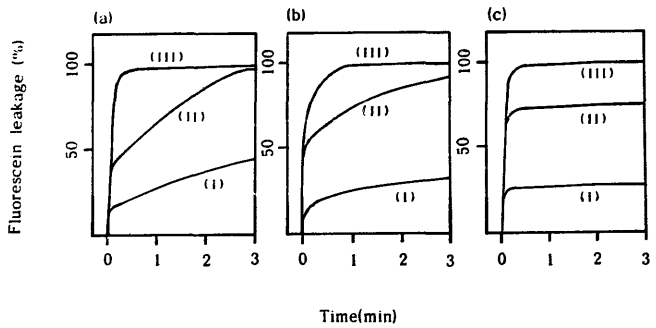

Fig. 5. Peptide-induced release profile along the time course of carboxyfluorescein encapsulated in egg PC-egg PG (3:1) liposomes with the addition of 4_3 (a), P-4_3 (b) or Amy-4_3 (c). Peptide concentrations were 0.8 μM (a-I), 1.8 μM (a-II), 3.0 μM (a-III), 0.8 μM (b-I), 3.5 μM (b-II), 4.5 μM (b-III), 1.0 μM (c-I), 3.2 μM (c-II) and 6.5 μM (c-III).

mum below 195 nm and a minimum at 214 nm in an aqueous solution, close to the values of 195 and 218 nm obtained for poly(L-lysine) in the β-form in an aqueous solution. The spectra measured in acidic and neutral liposomes showed a CD band of a double minimum at 206 and 222 nm, which is characteristic of the α-helix. Estimation of secondary structural parameters at various conditions is listed in Table I.

On the other hand, P-4_3 took an α-helical structure in an aqueous solution and in the presence of liposomes (Fig. 3b). Peptide 4_3 takes a random structure in the buffer solution and an α-helical structure in neutral and acidic liposomes as reported previously [6]. Therefore, formations of a β-structure and an α-helical structure in the aqueous solution of Amy-4_3 and P-4_3, respectively, seem to be induced by the strong mutual hydrophobic interaction of the long alkyl chains attached to 4_3.

To attain further understanding of the conformational change mediated by the peptide–lipid interaction, the binding of Amy-4_3 to the lipid bilayer was studied as a function of lipid concentration (Fig. 4). At a ratio of lipid/peptide of 3, the pattern of the β-structure of the peptide disappeared. At a ratio of 20, it took an α-helical structure and change in the helical contents was no longer observed at higher ratios. These results indicate that the β-sheet structure was disaggregated at a low ratio of lipid/peptide and the α-helical structure was induced and stabilized at a slightly higher ratio.

Leakage of liposome contents

To evaluate the interaction between peptides and model membranes, the ability of the peptides to leak encapsulated carboxyfluorescein from egg PC-egg PG (3:1) vesicles was measured. Dilution of carboxyfluorescein initially encapsulated at a high self-quenching concentration in the vesicles, resulted in a large enhancement in fluorescence intensity. Since dilution of carboxyfluorescein will arise only if the vesicles are rendered leaky, this enhancement in fluorescence intensity reflects the modification of vesicles by the peptides. Fig. 5a-c shows the release profile along the time course of carboxyfluorescein from egg PC-egg PG (3:1) vesicles with the addition of 4_3, P-4_3 or Amy-4_3. Fig. 6 shows the leakage ability as a function of peptide concentration. Peptides 4_3 and P-4_3 leaked the dye gradually with increasing peptide concentration (Fig. 5a, b) and reached maxima at the concentrations of 3 μM and 5 μM (Fig. 6), respectively. On the other hand, Amy-4_3 caused an initial rapid release of the dye and this release soon reached a plateau (Fig. 5c). The 100% dye release concentration was 6 μM (Fig. 6).

These results indicate that the order of the ability of peptides to release dye is 4_3 > P-4_3 > Amy-4_3. However, as observed in Fig. 5a-c , the interaction mode with lipids was different between peptides 4_3 and P-4_3 and peptide Amy-4_3.

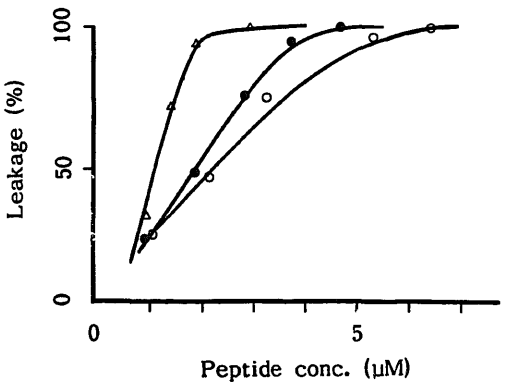

Fig. 6. Peptide-induced release of carboxyfluorescein encapsulated in egg PC-egg PG (3:1) liposomes as a function of the peptide concentration. The data were collected at 3 min after incubation of the peptide in liposomes, 4_3 (△), P-4_3 (●), Amy-4_3 (○).

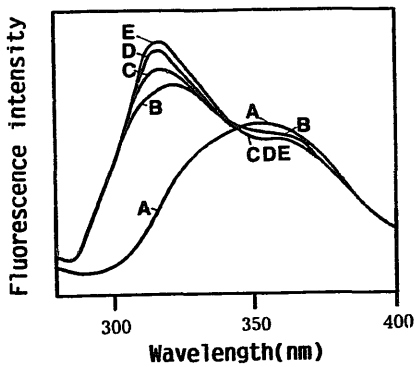

Fig. 7. Fluorescence emission spectra of Amy-4_3 in egg PC-egg PG (3:1) liposomes as a function of the lipid concentration. Peptide concentration was 10 μM. Lipid/peptide: A (peptide only), B (5), C (10), D (20) and E (40).

Fluorescence study of Amy-4_3

To attain further information on the peptide–lipid interaction in the lipid bilayers, the interaction of Amy-4_3 with egg PC-egg PG liposomes (3:1) was examined by fluorescence spectroscopy. As shown in Fig. 7, the emission spectrum of Amy-4_3 in the buffer solution gave a maximum peak at 357 nm with a shoulder around 330 nm. In the presence of egg PC-egg PG, a new maximum appeared at 318–330 nm which is probably attributable to the blue shift of the shoulder peak observed at about 330 nm in the buffer. The maximum peak showed an enhancement of fluorescence intensity. It is probable that the blue shift reflects the incorporation of the peptide into the lipid bilayer. However, a slight decrease in the maximum of 357 nm without any shifting suggested that a part of the peptide still remained in the hydrophilic environment.

It is probable that since Amy-4_3 takes a β-structure in the buffer solution and mainly an α-helical structure in the liposomes as observed in the CD experiment, the spectral change reflects the conformational change from the β-structure in aqueous solution to the α-helical structure in the lipid bilayer.

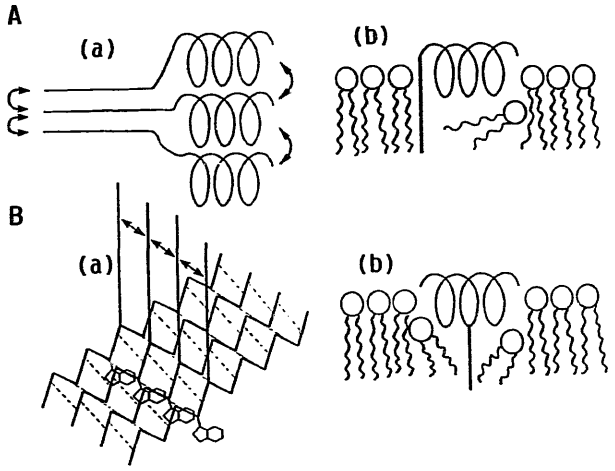

Fig. 8. Schematic representation of the orientation of P-4_3 (A) and Amy-4_3 (B) in aqueous solution(a) and liposomes(b). ——— represents hydrophobic interaction and - - - - - - represents hydrogen bond in the β-sheet structure.

Discussion

Fatty acids covalently attached to proteins have been found to acylate an amino-terminal residue or modify cysteine or threonine in proteins. In the present study, amphipathic α-helical peptides with a long alkyl chain were designed and synthesized as models for investigating the conformation of peptides and their anchoring to the lipid bilayer. Although an N-terminal myristoylated peptide and an internal O- or S-palmitoylated one are desirable as model peptides, P-4_3 and Amy-4_3 were at first selected because of their relatively easy preparation. In an earlier paper [6], an amphipathic α-helical peptide 4_3 was subjected to the study of lipid–peptide interaction. This peptide mainly takes a random structure in a polar solvent and an α-helical structure in liposomes. In the present study, P-4_3, which is a model of peptides with a covalently attached alkyl group at the N-terminus, took an α-helical structure in both media, indicating that this peptide exists in the aggregated state in the buffer and in the immersed monomer state in the lipid bilayer. On the contrary, Amy-4_3 took a β-structure in the buffer and an α-helical structure in the presence of lipids. The fluorescence study suggests that the Trp residue of Amy-4_3 exists in the hydrophobic surrounding in the presence of liposomes, i.e., the peptides are incorporated into the lipid bilayer.

Schematic representation of the interaction of the model peptides with the lipid bilayer is proposed in Fig. 8, from the results of CD and fluorescence studies. We previously reported that 4_3 exists as a mainly unordered structure and a partially α-helical structure in an aggregated state in the buffer. Peptide 4_3 lies parallel to the surface of the lipids, where the hydrophobic region of 4_3 is shallowly buried in the amphipathic moiety of the bilayer [8]. Peptide P-4_3 (Fig. 8A) was present as an α-helical structure in the buffer, suggesting that the N-terminal long acyl chains aggregate with strong mutual hydrophobic interaction, followed by the induction of the α-helical structure in the peptide part of the molecules. In the lipid bilayer, both the acyl chain and the hydrophobic part of the α-helix in the peptide interact with the hydrophobic part of the membrane by penetration as proposed for 4_3. The higher helical content in the acidic liposomes than in the neutral liposomes, as observed in the CD experiment, means that the charge interaction between the hydrophilic part of the peptides and the phospholipid head group helps preferentially their interaction. On Amy-4_3 (Fig. 8B), intermolecularly strong hydrophobic interaction of the Amy residue induces the β-structure for the peptide backbone in the buffer. The Trp residue in the Amy-4_3 in this conformation exists at the hydrophilic side with

a neighboring cation of arginine. This conformation was considered likely, due to the fact that the emission spectrum of Trp in the buffer indicated the presence of Trp in a highly polar environment. However, in the lipid bilayer, since the alkyl chain of Amy interacts with the alkyl chain of the lipid bilayer, the strong intermolecular interaction of the β-structure is lost, resulting in the incorporation of the peptide into the lipid bilayer and the formation of an α-helical structure.

The dye-leakage experiment, which evaluates the membrane-modifying effect of the peptides, indicated that the order of their perturbative abilities against egg PC-egg PG (3 : 1) liposomes was 4_3 > P-4_3 > Amy-4_3. It is likely that the long alkyl chain in the peptides which penetrated into the lipid bilayer acts as a constituent of the bilayer to make the bilayer stable. This speculation is consistent with the previous result that P-4_3 fluidized the DPPC-liposomes, thus producing no phase transition of the lipids [11]. On the release profile of carboxyfluorescein as a function of time, however, 4_3 and P-4_3 obviously increased the dye release gradually, whereas Amy-4_3 initially released the dye but then the release showed no further increase. The difference in these release profiles may be due to some differences in the action modes between peptides 4_3 and P-4_3 and peptide Amy-4_3 concerning the lipid bilayer. This is to say, Amy-4_3 interacts with the lipid bilayer to penetrate rapidly by perturbing it and then stabilizes the bilayer according to the action mode of Fig. 5c. Peptides 4_3 and P-4_3 interact gradually with the lipid bilayer to perturb it or else have a weaker stabilizing effect than Amy-4_3.

It is generally accepted that peptides and proteins bind to the polar surface of the lipid bilayer or else penetrate completely or to some extent into the interior of it, when they interact with the biomembrane. The fatty acid covalently attached to peptides and proteins may promote the anchoring of them to the lipid bilayer. However, the conformational feature of the membrane-anchoring domains has not been directly studied. The present study concerning model peptides suggests that the long alkyl chain group attached to peptides and proteins penetrates into the lipid bilayer and induces a suitable conformational change to show their function.

Myristoylated or palmitoylated proteins are often found in nature. Interestingly, myristic acid is only found attached to the N-terminal and palmitic acid is found in the body of polypeptides, although it is unknown why there is such a difference in terms of localization of these fatty acids. In the present study, we showed that the amphipathic α-helical peptides with a long alkyl chain attached to the N-terminus and in the middle of the sequence, interacted with the lipid bilayer in a different mode. Thus, lipid modification which is required to show the function of peptides and proteins may have some inherent sense according to the position of modification.

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