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Biochimica et Biophysica Acta 1283 (1996) 37–44

BBA Biochimica et Biophysica Acta

Fusion of liposomes due to transient and lasting perturbation induced by synthetic amphiphilic peptides

Jinbao Zhao, Shunsaku Kimura, Yukio Imanishi *

Department of Polymer Chemistry, Kyoto University, Yoshida Honmachi, Sakyo-ku, Kyoto 606-01, Japan

Received 1 April 1996; accepted 11 April 1996

Abstract

The membrane-fusion activities of amphiphilic peptides of H-(Leu-Aib-Lys-Aib-Aib-Lys-Aib)$_n$-Ala-N($C_{18}H_{37}$)$_2$ (n = 1, P7D and n = 3, P21D) immobilized on liposome were investigated. P7D, which takes a random conformation, induced fusion of DPPC SUV, but P7D immobilized on the DPPC SUV did not show the fusion activity. On the other hand, P21D showed a high activity of membrane fusion either in the free peptide or in the immobilized state. CF-Leakage experiments revealed that the peptides caused a transient perturbation of the membrane structure on binding to the membrane. A lasting and steady perturbation was also caused by P21D embedded in the membrane, which was indicated by Eu^{3+} permeation through the membrane. This type of membrane perturbation was very slight in the case of P7D embedded in the membrane. A conclusion was reached that the different activities in the membrane fusion are based on the transient perturbation in the membrane at the peptide binding to the membrane surface as well as the steady perturbation caused by the peptide embedded in the membrane.

Keywords: Membrane fusion; Amphiphilic peptide; α-Helix; Membrane perturbation; Peptide/lipid conjugate

1. Introduction

Membrane fusion can be divided into three steps: (i) association of two bilayer membranes, (ii) perturbation of the bilayer structure to form fusion pore at the contact point, and (iii) dilatation of the fusion pore [1–3]. Although step i has been clarified in the fusion of synaptic vesicles with the active zone of the synapse [4,5], the molecular mechanism of step ii has not been made clear. The fusion activities of a number of synthetic amphiphilic helical peptides have been investigated. For example, poly(Lys-Aib-Leu-Aib) induced fusion of DPPC vesicles more intensively at pH 9.0 than at pH 6.3 [6]. The enhancement of fusion activity at pH 9.0 was attributed to increase of α-helix content of the peptide upon deprotonation of the N-terminal α-ammonium group. In general, the amphiphilic α-helical peptide binds strongly to the bilayer membrane, thereby disturbing the membrane structure [7]. The fusion activity of peptides has been discussed in terms of orientation of the helical peptide in the membrane [8] or uneven distribution of side-chain bulkiness around the helix [9]. In either discussion, however, the ability of peptide to perturb membrane structure is a common key factor for membrane fusion.

Amphiphilic peptides in membrane are in dynamic equilibrium with those in aqueous solution. Therefore, the membrane structure may undergo a transient and drastic perturbation upon binding to or dissociation from the membrane of the peptide, and a lasting and steady perturbation by the peptide embedded in the membrane. The both types of perturbations have been detected in the peptide-induced leakage of CF from liposome. The release pattern is generally composed of two stages, an abrupt release immediately after the addition of peptides and a

Abbreviations: Aib, 2-aminoisobutyric acid; ATR-IR, attenuated total reflection-infrared; CD, circular dichroism; CF, 5(6)-carboxyfluorescein; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; MLV, multilayer large vesicle; N-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; P21D, H-(Leu-Aib-Lys-Aib-Aib-Lys-Aib)$_3$-Ala-N($C_{18}H_{37}$)$_2$; P21D-DOPC, P21D immobilized on DOPC SUV by sonication; P21OMe, H-(Leu-Aib-Lys-Aib-Aib-Lys-Aib)$_3$-OCH_3; P21OMe-DOPC, the sonicated dispersion of P21OMe and DOPC; P7D, H-Leu-Aib-Lys-Aib-Aib-Lys-Aib-Ala-N($C_{18}H_{37}$)$_2$; P7D-DOPC, P7D immobilized on DOPC SUV by sonication; P7OBz, H-Leu-Aib-Lys-Aib-Aib-Lys-Aib-OBzl; SUV, small unilamellar vesicle

* Corresponding author. Fax: +81 75 753-4911.

PII S0005-2736(96)00071-5

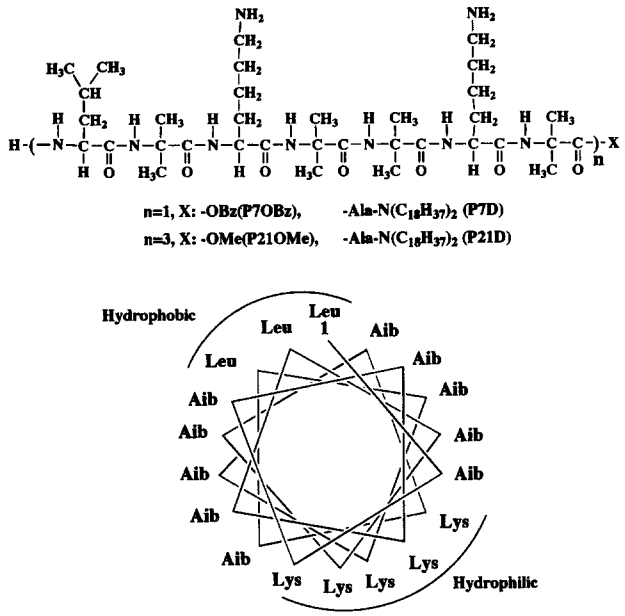


Fig. 1. Chemical structure of peptides synthesized and a helical wheel of P21D.

subsequent moderate release [10]. The initial leakage is ascribed to a transient perturbation in membrane structure upon binding of peptide, and the following slow leakage to a lasting perturbation due to the peptide embedded in the membrane. Therefore, the membrane fusion induced by amphiphilic peptides should be discussed in terms of the transient and the lasting perturbations in membrane produced transiently and steadily by the peptides.

Hydrophilic compounds conjugated with two long alkyl chains can be immobilized on liposome [11,12]. It has been reported that substantial intermembrane transfer of hydrophilic macromolecules conjugated with diacyl lipids takes place on a time scale of several hours. In the present study, H-(Leu-Aib-Lys-Aib-Aib-Lys-Aib)$_3$-Ala-N($C_{18}H_{37}$)$_2$ (P21D) was synthesized as a fusogenic peptide. The dialkyl peptide was designed to form amphiphilic α-helical structure and to make it difficult to dissociate from the membrane (Fig. 1). The primary sequence of P21D was so designed that the belt-shaped hydrophilic and hydrophobic surfaces align along the helix axis to result in highly amphiphilic property compared with poly(Lys-Aib-Leu-Aib) reported before as a fusogenic peptide [6].

2. Materials and methods

2.1. Materials

H-Leu-Aib-Lys-Aib-Aib-Lys-Aib-OBzl (P7OBz), H-Leu-Aib-Lys-Aib-Aib-Lys-Aib-Ala-N($C_{18}H_{37}$)$_2$ (P7D), P21D and H-(Leu-Aib-Lys-Aib-Aib-Lys-Aib)$_3$-OCH_3 (P21OMe) were prepared by the usual method in solution and their synthesis was confirmed by thin layer chromatography, ^{1}H-NMR, amino acid analysis and elemental analysis as reported previously [13].

Dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC) and 5(6)-carboxyfluorescein (CF) were purchased from Sigma Chemical, USA. *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (N-NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)-phosphatidylethanolamine (N-Rh-PE) were obtained from Molecular Probe, USA. All other reagents were of highest purity available.

2.2. Membrane fusion

Membrane fusion was assayed by the probe dilution method [14–16]. A chloroform solution of N-NBD-PE (0.5 mol%), N-Rh-PE (0.5 mol%) and DOPC or DPPC (25 mg) was evaporated to form a thin film. The dried film was dispersed in a Tris buffer solution (3.0 ml, 10 mM Tris, 0.1 mM EDTA; pH 7.4). The dispersion was sonicated with a Tomy UR-200P ultrasonic disrupter for 10 min under N_2 atmosphere. The vesicles were subjected to ultracentrifugation at 100 000 × *g* to obtain probe-containing SUVs. Phospholipid concentration was determined by a colorimetric assay using Diacolor-PL (Toyobo, Japan), and the stock solutions of SUVs were adjusted to be 10 mM of lipids. Peptide-containing SUVs were prepared from a chloroform solution of the peptides and DOPC followed by a procedure similar to that described above. The probe-containing SUVs (and the probe-free DPPC SUVs when specified) were dispersed in a Tris buffer solution, and an ethanol solution of peptide (P7D, P21D, or P21-OMe, 1.5–2.0 mM) or peptide-containing SUV was added. The volume of ethanol solution added to the dispersion was kept less than 1.0 vol% of the total volume. The peptide/lipid ratios in the peptide-containing vesicles were the same as those in the preparation of vesicles, because P21D and P7D were shown to be taken up completely by SUVs upon sonication of the dispersions [17]. The mixture was excited with a 450-nm light and the fluorescence intensity was measured at 530 nm (N-NBD-PE) and 593 nm (N-Rh-PE) with a Hitachi MPF-4 fluorophotometer at 30°C. The fluorescence intensity from N-NBD-PE was not changed by the presence of the peptides. On the other hand, N-Rh-PE fluorescence was slightly quenched by the peptides, but the quenching extent was less than 3.5% under the experimental conditions. *R* values will be affected by the quenching, but the change in *R* value is less than 1.8% at 50% fusion. Since the effect of the quenching on *R* values is very small, we did not compensate the *R* values. All the lipid concentrations specified are those for the SUV suspensions.

2.3. CF Leakage

CF-encapsulating DPPC SUVs were prepared according to the method reported by Barbet et al. [18]. A dry thin

membrane of DPPC or a mixture of DPPC and peptide (P7D or P21D) was dispersed in a Tris buffer solution (10 mM, pH 7.4) containing CF (0.1 M), and the mixture was sonicated and centrifuged to obtain SUVs. Free CF was removed by eluting through a Sephadex G-50 column using a Tris buffer solution (10 mM, pH 7.4) as eluant. CF-Encapsulating SUVs were dispersed in Tris buffer (10 mM (pH 7.4)), and an ethanol solution of peptides or peptide-containing SUV was added. In the cases of CF-encapsulating SUV composed of DPPC and the peptide, fluorescence intensity was monitored immediately after elution from the column. Zero leakage and complete leakage were determined from the fluorescence intensity of CF at time zero and in the presence of Triton X-100 (0.5 wt.%), respectively. The excitation and monitoring wavelengths were 470 and 520 nm, respectively. The temperature was controlled at 30°C, and the concentration of DPPC was 0.125 mM.

2.4. Eu^{3+} Permeation

DPPC SUVs or peptide-containing SUVs in deuterium oxide containing lanthanum nitrate (10 mM) were prepared by the sonication method [19–21], and were subjected to 270 MHz ^{1}H-NMR measurements (JEOL 270) at 50°C. The dispersion was kept at 50°C for 20 min and then europium nitrate (1 M) in a deuterium oxide solution was added to the SUVs to a final concentration of 10 mM. The intensities of two signals of the choline methyl groups of DPPC SUVs were measured at different times to evaluate the Eu^{3+} permeation.

2.5. ATR-IR Measurement

ATR-IR Spectra were recorded on a Nicolet Model 710 Fourier transform infrared spectrophotometer equipped with an MCT detector [22]. Peptide-containing MLVs were prepared from a dispersion in deuterium oxide of P21D (0.20 mM) and DMPC (20 mM) by a freeze-thaw method. The dispersion was eluted on a Ge plate to cast a film. The thin film was hydrated with deuterium oxide for 5 min. The ATR-IR measurements were carried out under N_2 atmosphere saturated with deuterium oxide vapor at 26°C.

3. Results

3.1. Fusion induced by free peptides

The extent of fusion of liposome was estimated by the probe dilution method. The excitation energy transfer from N-NBD-PE to N-Rh-PE, both of which were dispersed in DOPC or DPPC liposome, is suppressed upon fusion with probe-free liposome due to dilution of the probes in the membrane. The extent of fusion was determined from a value of R, which is the ratio of fluorescence intensity of N-NBD-PE against that of N-Rh-PE, the R value at the complete mixing of lipid molecules being taken as 100%. A slow fusion of membranes took place by addition of P21OMe, P21D or P7D, but scarcely occurred by P7OBz (Fig. 2). The fusion experiments were carried out under the same concentrations in amino-acid residues of the peptides, because the fusion activity of the peptides can be compared on the basis of the interaction of the residue with the lipid membrane (therefore, conformation of peptides), and may neglect the influence of the difference of molecular size of the peptides, which should be considered when the actual peptide concentrations were set at the same concentration.

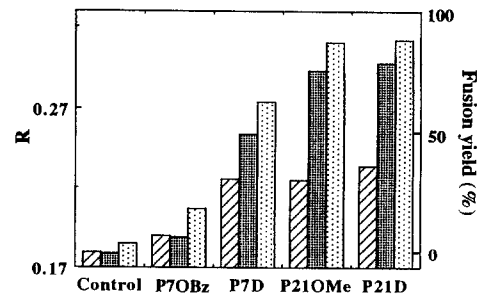


Fig. 2. The rate of membrane fusion induced by free peptides added to the dispersion of the probe-containing DOPC SUV ([DOPC] = 0.5 mM) and the probe-free DOPC SUV ([DOPC] = 1.0 mM) at 30°C. Incubation time with peptides: crosshatched bars, 0.25 h; shaded bars, 4 h; stippled bars, 8 h. [peptide] = 0.21 mM in amino-acid residue ([P7OBz] = 0.03 mM, [P7D] = 0.026 mM, [P21OMe] = 0.01 mM, [P21D] = 0.0095 mM]).

The initial velocity of fusion increases with increasing amount of probe-free SUVs (Fig. 3), suggesting that the fusion begins with collision of SUVs. The rate of fusion induced by P21OMe, P21D and P7D increased almost linearly with the peptide concentration (Fig. 4). Therefore, aggregation of peptides in membrane may not be important for the membrane fusion under the present conditions.

3.2. Fusion induced by peptides in membrane

A mixture of peptide and phospholipid was sonicated to obtain peptide-containing liposomes. P7D and P21D were

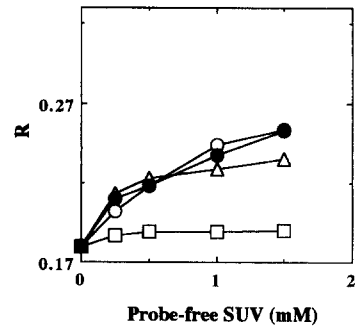


Fig. 3. The rate of membrane fusion induced by free peptides after 1 h incubation with various amounts of probe-free DOPC SUV. P7OBz (□), P7D (△), P21OMe (○) and P21D (●) were added to the dispersion of probe-containing DOPC SUV ([DOPC] = 0.5 mM) and probe-free DOPC SUV at 30°C. [peptide] = 0.21 mM in amino-acid residue ([P7OBz] = 0.03 mM, [P7D] = 0.026 mM, [P21OMe] = 0.01 mM, [P21D] = 0.0095 mM]).

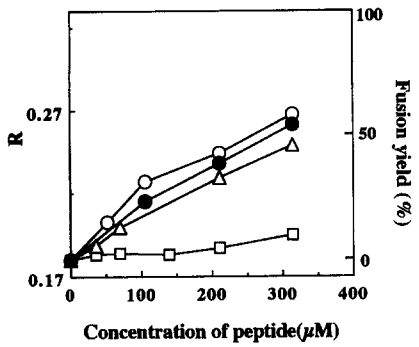


Fig. 4. Dependence of fusion rate on peptide concentration. The peptides (P7OBz (□), P7D (△), P21OMe (○) and P21D (●)) were added to the mixture of the probe-containing DOPC SUV ([DOPC] = 0.5 mM) and the probe-free DOPC SUV (0.5 mM). The extent of fusion (R) was determined after 1 h incubation of the peptide, the probe-containing DOPC SUV and the probe-free DOPC SUV. The peptide concentrations are expressed in amino-acid residue. The actual peptide concentrations were 1/7-fold for P7OBz, 1/8-fold for P7D, 1/21-fold for P21OMe, and 1/22-fold for P21D.

quantitatively partitioned to phospholipid membrane [17]. P7D-DOPC did not show fusion activity (Fig. 5), although free P7D induced the fusion of liposome (Fig. 3). P21OMe-DOPC induced a time-dependent fusion as free P21OMe did, the latter being faster. Probably, a part of P21OMe was taken into the inner aqueous phase of liposome, resulting in a decrease of P21OMe concentration in the outer leaflet of liposome. Interestingly, P21D-DOPC shows fusion activity with a high rate. The fusion induced by P21D-DOPC was examined in the presence of varying amounts of the probe-free DOPC liposome (Fig. 6a). The rate of fusion was high and nearly independent of the concentration of the probe-free liposome. This observation suggests that the fusion between P21D-DOPC and probe-immobilized liposome is predominating. On the other hand, the rate of fusion induced by P21OMe-DOPC increased

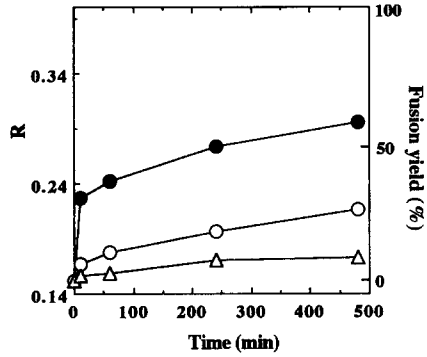


Fig. 5. The rate of fusion induced by peptide-containing DOPC SUV ([P7OBz] = [P7D] = [P21OMe] = [P21D] = 0.01 mM, [DOPC] = 1.0 mM). The peptide-containing DOPC SUVs (P7OBz-DOPC (□), P7D-DOPC (△), P21OMe-DOPC (○) and P21D-DOPC (●)) were added to the mixture of the probe-containing DOPC SUV ([DOPC] = 0.5 mM) and the probe-free DOPC SUV ([DOPC] = 1.0 mM). The extent of fusion (R) was determined after 1 h incubation of the peptide-containing DOPC SUV and the probe-containing DOPC SUV.

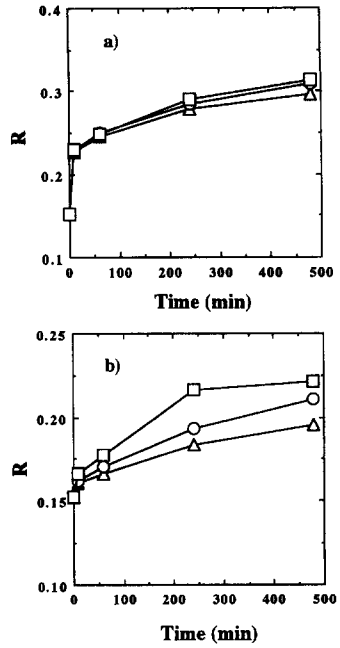


Fig. 6. The rate of fusion induced by (a) P21D-DOPC SUV and (b) P21OMe-DOPC SUV after 1 h incubation with various amounts of probe-free DOPC SUV. The peptide-containing DOPC ([P7OBz] = [P7D] = [P21OMe] = [P21D] = 0.01 mM, [DOPC] = 1.0 mM) was added to the mixture of the dispersion of the probe-containing DOPC SUV ([DOPC] = 0.5 mM) and the probe-free DOPC SUV ([DOPC] = (△) 0.5 mM; (○) 1.0 mM; (□) 1.5 mM) at 30°C.

with increasing concentration of probe-free liposome (Fig. 6b). It is considered that P21OMe-DOPC interacts either with the probe-containing liposome or with the probe-free liposome.

3.3. CF Leakage induced by free peptides

Perturbation in phospholipid bilayer membrane induced by peptides can be estimated by measuring CF leakage. The addition of P21OMe, P21D or P7D to CF-encapsulating DPPC liposome increased CF leakage in a concentration-dependent manner (Fig. 7). On the other hand, P7OBz did not accelerate CF leakage. Fig. 7 shows a fast leakage of CF immediately after the addition of P21OMe, P21D or P7D. The fast release is ascribed to binding of the peptides to liposome, thereby causing a transient perturbation in membrane structure. In the succeeding stages, the CF release by P7D was very slow, whereas it was moderate by P21OMe and P21D. The CF leakage in the latter stage should be due to a lasting and steady perturbation in the membrane by incorporation of peptide, because P21D and P7D are considered not to dissociate from the membrane [11]. On the other hand, P21OMe released CF in two stages, suggesting the presence of the transient perturbation as well as the lasting perturbation. Therefore, the effect of P21OMe on CF leakage is larger than that of P21D.

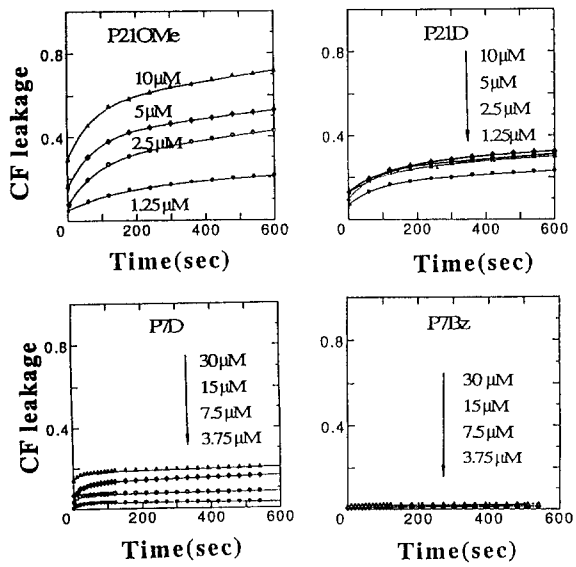


Fig. 7. CF leakage induced by free peptide added to CF-encapsulating DPPC SUV at 30°C. The ordinate represents the fraction of CF leaked from SUV taking complete leakage as unity. The actual peptide concentrations are designated in the figure. [DPPC] = 0.125 mM.

3.4. CF Leakage induced by immobilized peptides

The peptides were immobilized on liposome by the sonication method, and the peptide-containing liposomes were added to CF-encapsulating DPPC liposomes. The CF leakage is triggered by perturbation in CF-encapsulating liposome induced by peptides involved in other liposomes. It is clearly shown that P21D-DOPC and P7D-DOPC do not induce the CF leakage (Fig. 8), while free P21D and P7D induced it. The CF leakage induced by P21OMe-DOPC should be attributed to the ability of P21OMe to be redistributed to CF-encapsulating liposomes and to disturb the membrane structure.

The degree of perturbation of P21D-DOPC or P7D-DOPC membrane was estimated by CF leakage. A dry film of DPPC and P21D or P7D was dispersed in a Tris buffer solution containing CF (0.1 M). The dispersion was sonicated and eluted through a Sephadex G-50 column. It took 30 min from application of the dispersion to the column till the start of the fluorescence measurement. During this period, nearly half of CF entrapped in liposome was lost in the case of P21D. However, CF leakage during the preparation was low in the case of P7D. These results are shown in Fig. 9, indicating that P21D disturbs the membrane structure over a long period, much more than P7D. These results are consistent with the experiment of CF leakage by free P21D and P7D. The CF leakage due to the steady perturbation in membrane was observed in the case of P21D but not in the case of P7D.

3.5. Eu^{3+} Permeation through membrane

The lasting and steady perturbation in membrane induced by peptides can also be estimated by measuring Eu^{3+} permeation through the membrane. Two NMR peaks of the choline methyl groups of DPPC liposome appeared in the presence of Eu^{3+} in outer aqueous phase of liposome. One at a lower magnetic field is due to the choline methyl groups directing to the inside of liposome membrane, and the other at a higher magnetic field is due to the choline methyl groups in contact with the shift reagent of Eu^{3+}. The relative intensity of the two peaks of DPPC liposome was unchanged for more than 8 h of incubation at 50°C in the presence of Eu^{3+} in the aqueous phase (Fig. 10a), indicating the absence of membrane fusion and permeation of Eu^{3+}. The intensity ratio of the two peaks did not change in the presence of either P7D or P21D (1 mol%), indicating the absence of Eu^{3+} permeation across bilayer membranes. However, the peak at lower magnetic field disappeared after 5 h incubation in the presence of 1.3 mol% of P21D (Fig. 10b). On the other hand, 2.0 mol% of P7D in the membrane did not change the relative peak intensities in 5 h incubation (Fig. 10c). 4.0 mol% of P7D was necessary to affect the relative intensities. These results indicate that P21D perturbed the membrane structure more intensively than P7D.

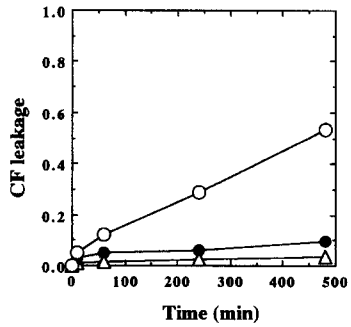


Fig. 8. CF leakage from DPPC SUV ([DPPC] = 0.125 mM) by the addition of P7D-DOPC (△), P21OMe-DOPC (○) and P21D-DOPC (●) SUVs at 30°C. [peptide] = 0.01 mM. [DOPC] = 1.0 mM. The ordinate represents the fraction of CF leaked from SUV taking complete leakage as unity.

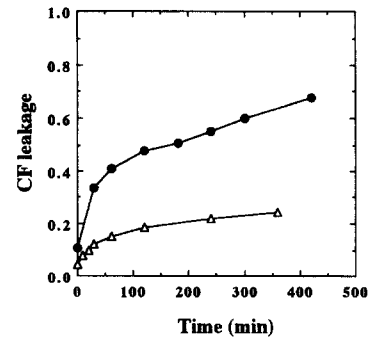
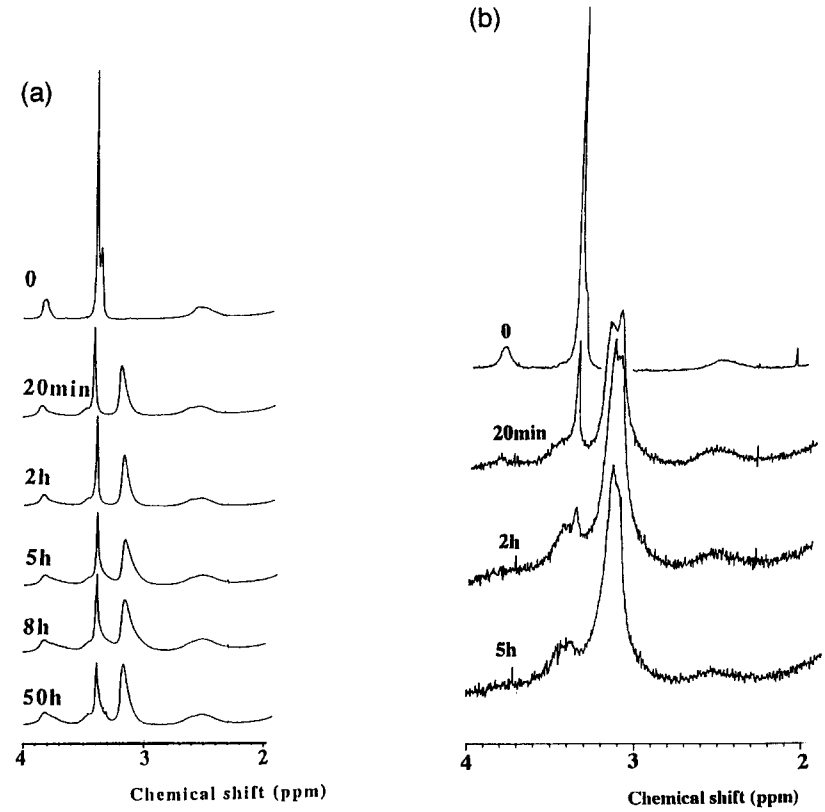


Fig. 9. CF leakage from P7D-DPPC (△) and P21D-DPPC (●) SUVs containing CF at 30°C. [DPPC]/[peptide] = 100. The ordinate represents the fraction of CF leaked from SUV taking complete leakage as unity.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Chemical shift (ppm)

3.6. Orientation of P21D in DMPC membrane

The interaction of P21D with phospholipid membranes was studied by FTIR-ATR. The dichroic ratio of amide I absorption of P21D changed by incubation in DMPC multilayer. It was 1.74 in a dry DMPC membrane, and 1.18 after incubation with D_2O for 5 min and exposure to hydrated N_2 atmosphere for 20 min. The average tilt angle of the helix axis of P21D calculated from the dichroic ratio is 60° and 90° before and after incubation, respectively [21]. The dichroic ratio of symmetric CH_2 stretching mode of DMPC was 1.08, indicating that DMPC multilayer is formed on the substrate and the average tilt angle is 25°. Therefore, the orientation of the helix axis is vertical to the bilayer normal in the hydrated DMPC membrane, while it is nearly random in the dry membrane.

4. Discussion

The peptides used here have positive charges aligning on one side of the helix rod. The repulsive electrostatic interactions between positive charges disturb the helical conformation, but helical conformation of P21OMe and P21D is stabilized by Aib residues, resulting in amphiphilic helical structure. The interaction of these peptides with anionic lipid bilayer membranes may be strong enough to cause membrane fusion faster than with neutral phospholipid bilayer membranes. In the present investigation, however, neutral membranes were used to slow down the fusion rate for investigation on the mechanism of peptide interaction with membrane in detail. Various types of peptides were investigated: P7OBz which does not interact with membrane, P21OMe which is distributed between lipid membrane and aqueous phase, P21D-DOPC which is a membrane-immobilized peptide with helix conformation, and P7D-DOPC which is a membrane-immobilized peptide with random conformation.

Membrane perturbation is considered to be prerequisite for membrane fusion, and can be induced by interactions with peptides. The CF-leakage profile is composed of two phases. One of them is fast release due to a transient perturbation in membrane caused by peptide binding, and the other is a slow leakage due to a lasting and steady perturbation. The transient perturbation was observed by the addition of P21OMe, P21D or P7D. Therefore, peptides are considered to perturb membrane structure upon binding to bilayer membranes.

P7D induced the initial fast release only, while P21D induced both the initial fast release and the later slow release. Since P7D and P21D do not dissociate from the membrane under the experimental conditions, the helical conformation of P21D in the membrane should perturb the membrane structure more strongly than the random conformation of P7D. This consideration is supported by the fact that CF leakage induced by P21D was larger than that by P7D at 3-fold concentration of P21D, where the leakage was compared by concentrations in amino-acid residues (Fig. 7). The experimental finding that the CF leakage was enhanced most intensively by P21OMe can be explained by the helical conformation of P21OMe in the membrane and a reversible binding to the membrane.

P7D induced membrane fusion. The extent of fusion in 15 min incubation with P7D was similar to that with P21OMe or P21D. Therefore, amphiphilic helical structure of peptides is not prerequisite for membrane fusion. The transient perturbation of membrane upon peptide binding should trigger membrane fusion.

The resonance energy transfer assay with N-NBD-PE and N-Rh-PE was used to demonstrate membrane fusion induced by the peptides. The fusion induced by the peptides was confirmed by another assay of dynamic light scattering (DLS) measurement. DOPC SUVs were prepared by a sonication method, and were treated by the extruder to homogenize the vesicle size [22]. The diameter of DOPC SUVs (originally 28.9 nm) became larger by 1 h incubation with P7D, P21D, or P21OMe to be 33.5 nm, 33.9 nm, or 35.7 nm, respectively, although it was not changed in the absence of the peptides in 1 h incubation. The diameter will become 41 nm upon fusion of two SUVs under the assumption that the occupied area of one lipid in the membrane does not change before and after fusion. The results of DLS measurements suggest that the fusion induced by the peptides occurs between SUVs. Therefore, one of the driving forces of the membrane fusion may be due to defects produced in a large curvature of the membrane.

In respect of the membrane fusion ability of the peptides immobilized on SUVs, P21D-DOPC was significantly high, while P7D-DOPC was negligible (Fig. 5). The CF leakage and the Eu^{3+} permeation experiments showed that the membrane structure was more strongly disturbed in P21D-DOPC than in P7D-DOPC. The high fusion activity of P21D-DOPC may, therefore, be ascribed to the amphiphilic helical structure of P21D. However, the size difference between P21D and P7D may explain the different fusion ability of the peptides. It was difficult to examine this point experimentally because the membrane was significantly disturbed at higher peptide concentrations ([lipid]/[peptide] < 100) than that expected from the perturbation at lower peptide concentrations ([lipid]/[peptide] > 100). Probably, aggregation of peptides in the membrane will occur at high peptide concentrations, resulted in large membrane perturbation.

Fig. 10. ^{1}H-NMR spectra of DPPC SUV at 50°C in the absence of peptides (a), in the presence of (b) 1.3 mol% of P21D and (c) 2.0 mol% of P7D. The concentrations of both lanthanum nitrate and europium nitrate in a deuterium oxide solution were 10 mM.

The R value in the presence of free P21OMe or P21D increased with increasing amount of the probe-free SUV (Fig. 3). This is because the peptides are distributed to the probe-free and the probe-containing SUVs to increase the amount of SUVs having defects in membrane. However, the increase of R value by P21D-DOPC was independent of the concentration of the probe-free SUV. The peptides immobilized on SUVs did not strongly perturb the membrane structure of other SUVs (Fig. 8). ATR-IR measurement revealed that the peptide part of P21D oriented itself in parallel to the bilayer surface. Therefore, the peptide part of P21D is incorporated tightly into the surface region of membrane and hindered from strong contact with other SUVs. In other words, the fusion activity of P21D-DOPC is sensitive to the membrane structure of the other SUVs coming into contact for fusion. It is, therefore, considered that P21D-DOPC should undergo fusion only with the probe-containing SUVs, which possess membrane defects due to a large fluorescent probes. However, we can not exclude the possibility that positively charged peptides on DOPC SUVs may strongly interact with negatively charged N-NBD-PE and N-Rh-PE of the probe-containing DOPC SUVs, resulted in fusion between these SUVs.

To conclude, the present study clearly shows that the fusion activity of peptides is not simply related to the amphiphilic helical conformation of the peptides. The fusion activity of the peptides should be considered from the following two points: (i) a dynamic binding equilibrium with lipid membrane, and (ii) a lasting and steady perturbation of lipid membrane in which they are incorporated or with which they come into contact. With respect to the first point, peptides induce membrane fusion by the perturbation in membrane produced either at binding process or at dissociation process, and hence fully hydrophobic or hydrophilic peptides will possess low fusion activity. On the other hand, amphiphilic peptides, which possess a moderate affinity for phospholipid bilayer membrane, have a strong fusion activity. With respect to the second point, amphiphilic helical peptides possess a strong fusion activity due to intensive perturbation produced in lipid membrane by the embedded peptide. However, the amphiphilic helical peptides are incorporated on the lipid membrane so tightly that they do not disturb the structure of other membranes coming into contact. Consequently, their fusion activity is lowered. Therefore, peptides of the ideal amphiphilic helicity may not be effective fusogens [23]. The peptides with a moderate membrane affinity and an imperfect amphiphilic helicity may have a high fusion activity.

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