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Roles of peptide–peptide charge interaction and lipid phase separation in helix–helix association in lipid bilayer

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Abstract

The roles of peptide–peptide charged interaction and lipid phase separation in helix–helix association in lipid bilayers were investigated using a model peptide, P_{24}, as a transmembrane α-helical peptide, and its four analogues. Fluorescence amino acids, tryptophan ($P_{24}W$) and pyrenylalanine ($P_{24}Pya$), were introduced into the sequence of P_{24}, respectively. Association of these peptides permits the resonance excitation energy transfer between tryptophan in $P_{24}W$ and pyrenylalanine in $P_{24}Pya$ or excimer formation between $P_{24}Pya$ themselves. To evaluate the effect of charged interaction on the association between α-helical transmembrane segments in membrane proteins, charged amino acids, glutamic acid ($P_{24}EW$) and lysine ($P_{24}KPya$), were introduced into $P_{24}W$ and $P_{24}Pya$, respectively. Energy transfer experiments indicated that the charged interaction between the positive charge of lysine residue in $P_{24}KPya$ and the negative charge of glutamic acid residue in $P_{24}EW$ did not affect the aggregation of transmembrane peptides in lipid membranes. As the content ratio of sphingomyelin (SM) and cholesterol (Ch) was increased in the egg phosphatidylcholine (PC), the stronger excimer fluorescence spectra of $P_{24}Pya$ were observed, indicating that the co-existence of SM and Ch in PC liposomes, that is, the raft of SM and Ch, promotes the aggregation of the α-helical transmembrane peptides in lipid bilayers. Since the increase in the contents of SM and Ch leads to the decrease in the content of liquid crystalline-order phase, the moving area of transmembrane peptides might be limited in the liposomes, resulting in easy formation of the excimer in the presence of the lipid-raft.

Keywords: α-Helical transmembrane peptide; Energy transfer; Liposome; Lipid phase separation; Raft; Charge interaction; Helix–helix interaction

1. Introduction

Since transmembrane helices are the most common secondary structural feature in membrane proteins, helix–lipid and helix–helix interactions must participate in the association and stabilization of membrane proteins (Ref. [1] as a review). Helix–helix in membrane proteins are held together by association factors that permit close packing among the helices by overcoming an entropy that favors helix separation. Possibilities for such association factors include hydrogen bonds, charge interactions, the interaction of helix dipoles, and the packing between lipid and protein [2]. In fact, it has been demonstrated that interhelical hydrogen bonding between two asparagine side chains contributes to oligomerization and stabilization of transmembrane α-helical peptides in lipid membranes [3,4]. However, there is little information about the effects of charged residues between helices on interhelical interaction.

The packing between lipid and helix must involve not only protein structure in membrane, but also the lipid phase behavior, especially the lipid fluidity. In this connection, the lateral organization of different lipid species in complex of lipid–protein has been recently found in biological membranes. The most profound organization has been found from studies on the transportation of transmembrane proteins governed by domains of sphingolipids and cholesterol (Ch). This domain, called "raft" [5], plays an important

Abbreviations: CD, circular dichroism; Ch, cholesterol; Fmoc-AA-OH, 9-fluorenylmethoxy carbonyl amino acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; PC, phosphatidylcholine; Pya, pyrenyl-L-alanine; SM, sphingomyelin; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TFA, trifluoroacetic acid

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part in not only signal transduction but also protein sorting [6,7]. These lipid-rafts can bind transmembrane proteins, such as influenza virus hemagglutinin [8] and neuramidase [9], and their primary structures of transmembrane domains involves the recognition events that form the basis for sorting membrane components in the biosynthetic pathway [10]. The oligomerization of transmembrane helix is also thought as one of the element to specify Golgi localization [11]. It is plausible to consider that the raft–peptide interaction plays an important role in the localization and oligomerization of transmembrane helices into cell membranes.

The present study was carried out to investigate the association factors of transmembrane protein, by focusing on: (1) the role of the charge interaction between helix–helix and; (2) the role of the lipid phase separation, especially of lipid assemblies produced by sphingomyelin (SM) and Ch binary mixture or SM and Ch in phosphatidylcholine (PC) bilayers. We chose a model peptide, P_{24}, which has been well characterized as a transmembrane

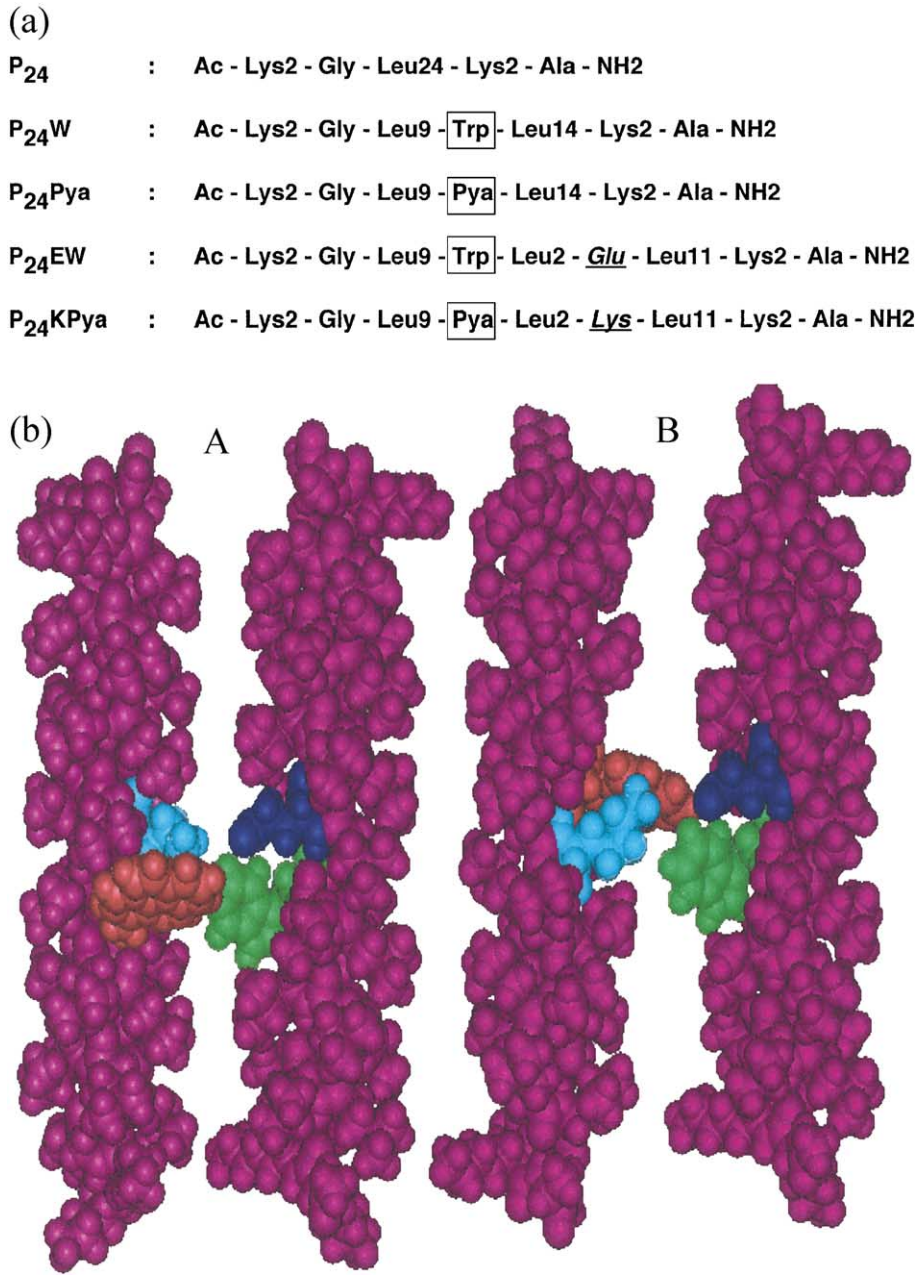

Fig. 1. (a) Primary structures of model peptide P_{24} and its analogues. (b) CPK model structures of the charged peptides present, which built up from parallel (A) or antiparallel (B) α-helical structures. When they are faced by the charged interaction, two Cα carbons of chromophore groups exist within about 13 and 14 Å. Trp (green), Pya (red), Glu (blue) and Lys (light blue).

α-helical peptide by studies of circular dichroism (CD), differential scanning calorimetry, low-angle X-ray diffraction, and H^1 and H^2 nuclear magnetic resonance [12–16]. Furthermore, to maintain the transmembrane orientation, the charged amino acid residues (lysine) were introduced on each terminal of the peptide. In the present studies, we designed and synthesized four analogues of P_{24}, which are expected as transmembrane α-helical model peptides (Fig. 1a). By using these analogues, we investigated the association factor and interaction mode of transmembrane peptides in phospholipid bilayers through CD, fluorescence studies and electron microscopy.

2. Materials and methods

2.1. Materials

The peptide synthetic reagents, 9-fluorenylmethoxy carbonyl amino acid (Fmoc-AA-OH), piperidine, diisopropylethylamine, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetrametyluromium hexafluorophosphate (HATU), thioanisole, 1,2-ethanditiol, *m*-cresole and trifluoroacetic acid (TFA) were purchased from Watanabe Chem. Ind. (Hiroshima, Japan). Fmoc-PAL-polyethylene glycol-polystyrene (Fmoc-PAL-PEG-PS) resin was supplied from PerSeptive Biosystems (Framingham, MA). Egg yolk PC (egg PC), bovine brain SM and Ch were obtained from Sigma (St. Louis, MO). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Wako (Osaka, Japan). All the reagents were used as received.

2.2. Peptide synthesis

All of the model peptides were synthesized according to the Fmoc chemical procedure started from Fmoc-PAL-PEG-PS resin (0.16 mmol/g) using a Pioneer Peptide Synthesizer (PerSeptive Biosystems) as described previously [17]. After the cleavage from the resin by anisole, 1,2-ethanditiol, *m*-cresole and TFA for 2 h at room temperature, the crude peptides were applied on Sephadex G-25 (25×130 mm) using 50% acetic acid. The peptides obtained from fractionation were purified by RP-HPLC using a C8 column (250×20 mm, Nacalai Tesque, Kyoto, Japan) with a gradient system of water/isopropanol containing 0.1% TFA. The main peaks were collected and their purity was also confirmed by RP-HPLC using an analytical C8 column (4.6×250 mm, Nacalai Tesque). Yields were about 25% for all peptides. Molecular mass was measured by the TOF-mass spectrum using a Voyager MALDI-TOF Mass spectrometer (PerSeptive Biosystems).

2.3. Liposome preparation

Mixture solutions of peptides and lipids (PC, PC–SM, PC–Ch, SM–Ch, or PC–SM–Ch) in chloroform/methanol (1:1) were evaporated under N_2 gas and the residual lipid films (including peptides) were dried under vacuum overnight. The films were hydrated with 2 ml of *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer solution (5 mM TES/100 mM NaCl, pH=7.4). The suspensions were vortexed and then sonicated for 10 min at 25 °C using a bath-type sonicator [18]. The unilamellar vesicles obtained were used for CD and fluorescence measurements.

2.4. CD measurements

CD measurements were performed using a JASCO J-600 spectropolarimeter with a quartz cell of 1 mm path length. Concentrations of lipids and peptides were 100 and 10 μM in TES buffer solution, respectively. The spectra were obtained from the average of eight runs of scanning. The CD data were collected by subtracting the spectra of lipid solutions from those of the lipid solutions containing peptides. CD values are expressed as the mean residue molar ellipticity. The α-helical contents (f_h) were calculated from the following equation :

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

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm. The values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, corresponding to 0 and 100% helical contents at 222 nm, are estimated to be −2000 and −30,000 deg cm^2 $dmol^{-1}$, respectively [19].

2.5. Fluorescence measurements

All fluorescence measurements were performed with a JASCO FP-777 fluorescence spectrophotometer. On energy transfer measurement, a liposome solution was excited at 285 nm. Concentrations of P_{24}Pya and egg PC were fixed at 1 and 100 μM, respectively. Concentrations of P_{24}W were prepared at 0, 1, 2, 4 and 8 μM. For charge interaction measurements, P_{24}Pya or P_{24}KPya as an acceptor peptide was mixed with P_{24}EW as a donor peptide under the same concentration of donor and acceptor peptides (1, 2, 3, 4 and 5 μM). On the measurements of P_{24}Pya excimer fluorescence spectra, concentrations of P_{24}Pya and mixed liposomes with different lipid compositions were fixed at 10 and 100 μM, respectively. The peptide–lipid mixed liposome solutions as described in Liposome preparation section were excited at 340 nm to observe the fluorescence of the pyrene excimer at 25 °C.

2.6. Electron microscopy

The peptide–lipid mixed films were hydrated in 5 mM TES buffer (pH=7.4) containing 100 mM NaCl as described in Liposome preparation section. The peptide

concentration was 50 μM and lipid concentration was 500 μM. Electron microscopy was performed by the negative-staining method as described previously [20]. After the sonication using a bath-type sonicator, the liposome solutions were left to stand for 24 h at 25 °C. The sample solutions were placed on the Formar carbon-coated grids and stained with 0.5% phosphotungstic acid adjusted to pH 7.4 with NaOH at room temperature. The liposomes were observed through a Hitachi HU-12A electron microscope (Hitachi, Hitachi, Japan).

2.7. Fluorescence anisotropy measurements

All measurements were performed with a Hitachi 850 fluorescence spectrophotometer. DPH dissolved in tetrahydrofuran was added to the liposome solution and then incubated at 25 °C for 1 h [21]. Two polarizers were set just front and behind the cell holder for excitation and emission, respectively. The excitation and emission wavelengths were 375 and 435 nm, respectively. The fluorescence anisotropy (r) was estimated based on the following definition

$$r = \frac{I_{//} - GI_{\perp}}{I_{//} + 2GI_{\perp}}$$

where $I_{//}$ and $I_{\perp}$ are the parallel and perpendicular components against the vertical excitation. G is the grating factor, which was determined by the intensity ratio of the parallel and perpendicular against the horizontal excitation.

3. Results

3.1. Design and synthesis of transmembrane α-helical model peptides

The 30-amino-acid model peptide, P_{24}, consists of 24 leucine residues as the transmembrane segment, which forms an α-helical structure [13]. Earlier reports show that the mismatch between bilayer hydrophobic thickness and peptide hydrophobic length generated different location of transmembrane peptides [11,16,22,23]. When the bilayer width exceeds the length of the hydrophobic segment, the mismatch induces the formation of a non-transmembrane orientation [22]. But P_{24} did not generate any such mismatch orientation, because the chain length of 24 leucine residues is much longer than that of the hydrophobic core of lipid acyl chains in bilayers [16]. To monitor the association mode of α-helical peptides in lipid bilayer, we introduced fluorescence amino acids, tryptophan and pyrenylalanine, into the sequence of P_{24}, respectively, which permit the resonance excitation energy transfer between tryptophan and pyrenyl-L-alanine (Pya), when both peptides come closer each other by the association.

Four analogues designed and synthesized on the basis of the transmembrane α-helical model peptide, P_{24}, are shown in Fig. 1a. $P_{24}W$ and $P_{24}Pya$ contain fluorescence amino acids, tryptophan and pyrenylalanine, in the center of transmembrane area of P_{24}, respectively. Furthermore, to evaluate the effect of charge interaction on the association between α-helical transmembrane segments in membrane proteins, charge amino acids, glutamic acid ($P_{24}EW$) and lysine ($P_{24}KPya$) were introduced into $P_{24}W$ and $P_{24}Pya$, respectively. The model structures of the charged peptides, which are built up from the two antiparallel or parallel α-helical structures and are present near the distance of van der Waals interaction, are shown in Fig. 1b. When they are faced by the charge interaction, two Cα carbons of chromophore groups exist at least within 13 and 14 Å.

3.2. CD studies

CD spectra were measured to determine the secondary structure of the peptides in lipid membranes. In the presence of egg PC (100 μM) liposomes containing peptides (10 μM), CD curves of all peptides showed typical double minimum bands at about 208 and 222 nm, indicating that the peptides took the α-helical structure in egg PC lipid bilayer. Their helical contents were about 75% (Fig. 2a).

Mixed lipid liposomes with various compositions of egg PC, SM and Ch containing $P_{24}Pya$ (10 μM) also showed typical double minimum bands (Fig. 2b). Their helical contents were not so different as compared with that in egg PC. Interestingly, the increase in the ratio of SM or/and Ch in egg PC liposome produced deeper at 208 nm.

3.3. Energy transfer experiments in liposomes

To investigate the association between two transmembrane α-helical peptides, we measured the energy transfer from $P_{24}W$ to $P_{24}Pya$ in the presence of egg PC liposomes. Fig. 3 shows fluorescence spectra of egg PC liposome (100 μM) containing 1 μM of $P_{24}Pya$ and various concentrations of $P_{24}W$. When $P_{24}W$ was excited at 285 nm in the absence of $P_{24}Pya$ in egg PC liposome, the fluorescence with a maximum at 332 nm of which lower energy tail was shown in Fig. 3. The fluorescence corresponding to pyrene chromophore is also observed at the absence of $P_{24}W$, because the pyrene chromophore has a weak absorbance at 285 nm. When $P_{24}Pya$ was mixed with $P_{24}W$ under the concentrations (1 μM), the spectrum corresponding to pyrene fluorescence was increased. Furthermore, the increasing concentrations of $P_{24}W$ led to increase in the intensity of pyrene fluorescence. These results demonstrate that the excitation energy of tryptophan moiety in $P_{24}W$ was transferred to the pyrene moiety in $P_{24}Pya$.

To study the effect of charge interaction on peptide aggregation in egg PC liposome, the efficiency of the energy transfer on the mixed system of $P_{24}EW$ and $P_{24}Pya$ was compared with that on system of $P_{24}EW$ and

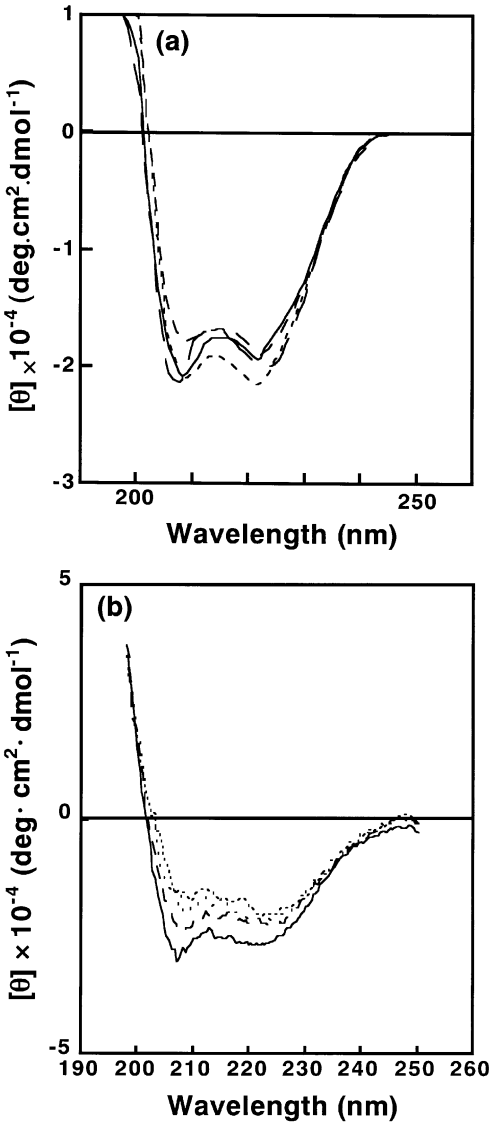

Fig. 2. CD spectra of P_{24} and its analogs in DPPC liposomes (a) and P_{24}Pya in various phospholipid liposomes (b). Peptide and lipid concentrations were 10 and 100 μM, respectively. (a) P_{24}W, (——); P_{24}Pya, (— —); P_{24}EW, (– – –); and P_{24}KPya (·····). (b) PC only, (——); PC/Ch (3:2), (— —); PC/SM (3;2), (– – –); and PC/SM/Ch (3/1/1) (·····).

P_{24}KPya. When the concentrations of both peptides were increased from 1 to 5 μM under the equivalent mixing-ratio of them, no remarkable difference in the fluorescence spectra was observed between both mixed systems (data not shown). When the intensities at 377 nm of each system were plotted as a function of the peptide concentrations (Fig. 4), the almost parallel lines were obtained and the values of slopes are only slightly larger on P_{24}EW–P_{24}KPya than on P_{24}EW–P_{24}Pya. These results indicate that the charge interaction between the positive charge of Lys in P_{24}KPya and negative charge of Glu in P_{24}EW does not contribute to the aggregation of transmembrane peptides in lipid membranes.

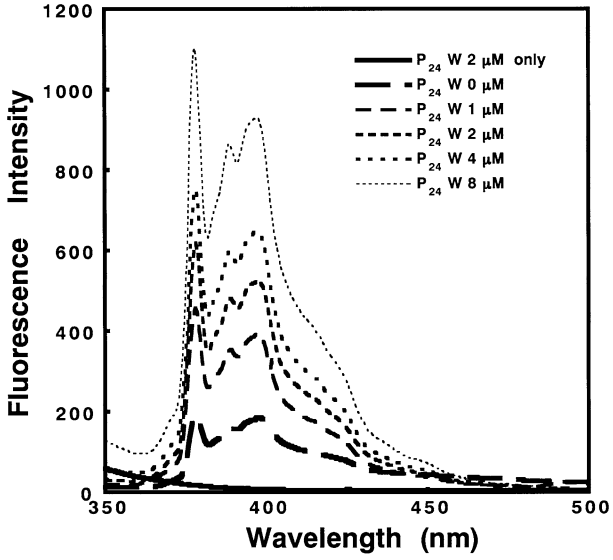

Fig. 3. The fluorescence spectra of P_{24}Pya by the energy transfer from P_{24}W in the presence of egg PC liposomes. Concentrations of P_{24}Pya and egg PC were fixed at 1 and 100 μM, respectively. The concentrations of P_{24}W were 0, 1, 2, 4, and 8 μM and the fluorescence of P_{24}W (2 μM) in the absence of P_{24}Pya is shown as peptide only. Excitation wavelength, 285 nm.

3.4. Aggregation of transmembrane peptides in mixed liposomes of egg PC with Ch and/or SM, and in binary mixture Ch and SM

The aggregation of α-helical transmembrane peptides in the mixed liposomes of the egg PC with Ch and/or SM in binary mixture of Ch and SM at different lipid compositions

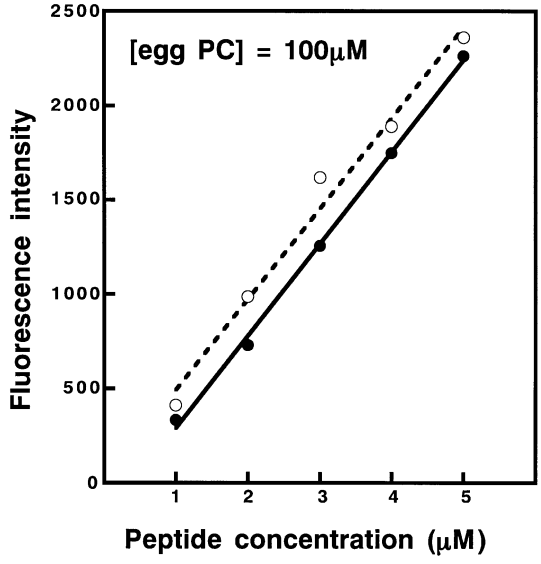

Fig. 4. Peptide concentration dependence of the fluorescence intensities by the energy transfer. The ratios of P_{24}EW against P_{24}Pya and P_{24}KPya were constant, 1:1. The excitation wavelength was 285 nm and therefore, the pyrenyl fluorescence intensity, due to the energy transfer was monitored at 377 nm. P_{24}EW–P_{24}Pya, (—●—); P_{24}EW–P_{24}KPya (- -○- -).

was measured using the fluorescence of pyrene in P_{24}Pya. It is well known that two pyrenes can form an excimer to emit the specific fluorescence around 450 nm, when two chromophores are present close together (within about 5 Å). Thus, the association of P_{24}Pya in lipid membrane was monitored by pyrene excimer formation. When P_{24}Pya was excited at 340 nm under the different peptide concentrations (5, 10, 20, and 30 μM) in egg PC liposomes (100 μM), no excimer was observed below 10 μM of peptide concentration (data not shown). By the addition of SM and/or Ch to the egg PC liposomes (total lipid concentration, 100 μM) containing a certain concentration of peptide (10 μM), no remarkable excimer fluorescence was also observed. Thus, peptide and lipid concentrations were fixed at 10 and 100 μM, respectively.

In the different ratios of PC/SM liposomes, no excimer peak was observed as shown in Fig. 5a. In the PC–Ch liposomes, a trace of excimer peak was observed at Ch concentrations of 20 μM and it became much larger at 30 μM. These results indicate that the peptides come closer when Ch concentrations were increased into PC liposome (Fig. 5b). Furthermore, when SM was added in the mixed system of PC–Ch, the excimer formation yield became much larger with increasing ratios of SM than that of the PC–Ch (Fig. 5c). For instance, the fluorescence intensity of the excimer in PC–SM–Ch (4/3/3) at 450 nm is at 1.5 as

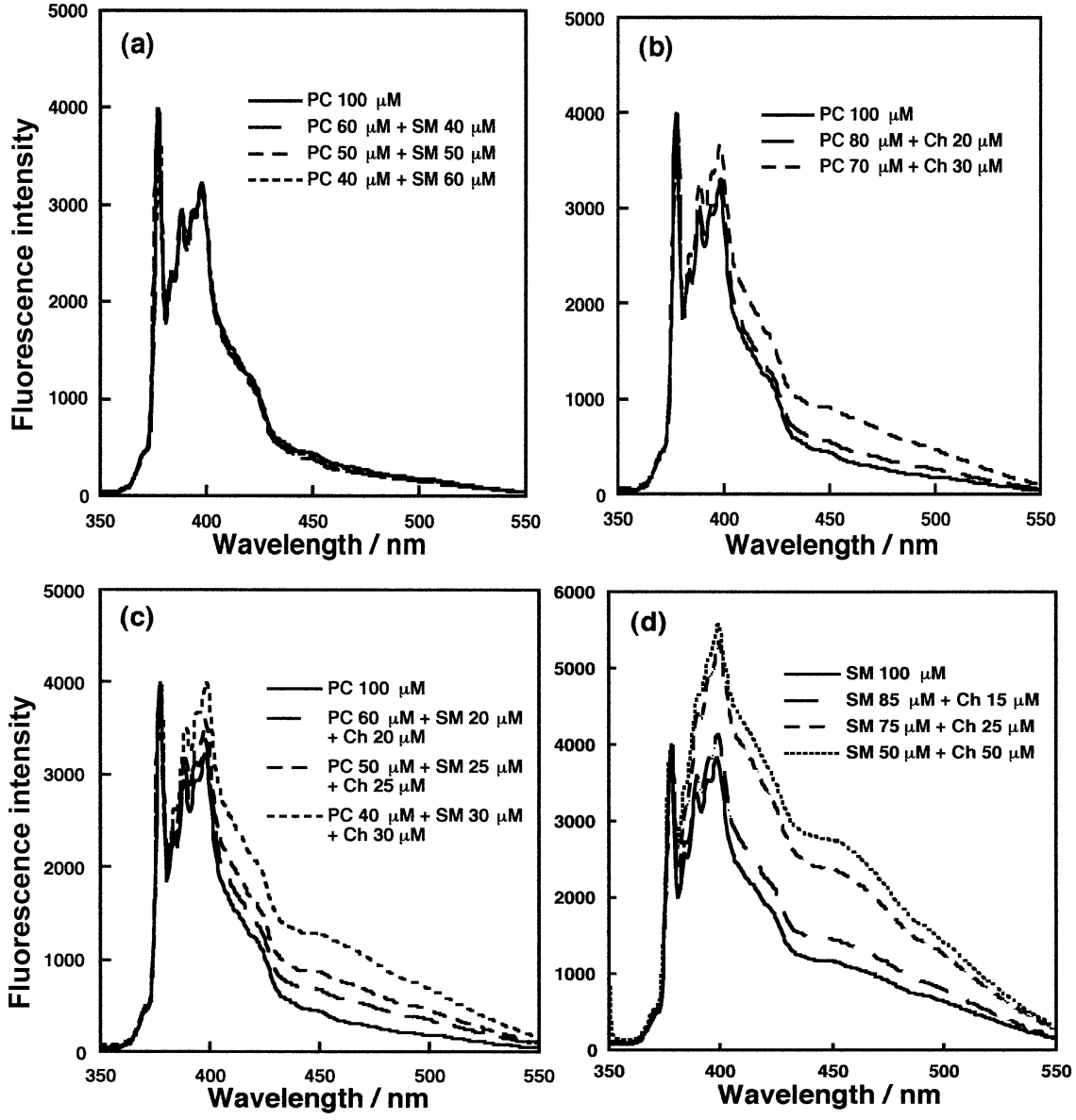

Fig. 5. Fluorescence spectra of P_{24}Pya in different lipids and lipid compositions: (a) PC–SM liposomes; (b) PC–Ch liposomes; (c) PC–Ch–SM liposomes; (d) Ch–SM liposomes. Peptide and lipid concentrations were at 10 and 100 μM, respectively. Liposome solutions were excited at 285 nm and the fluorescence emission spectra were normalized to the peak maximum intensity.

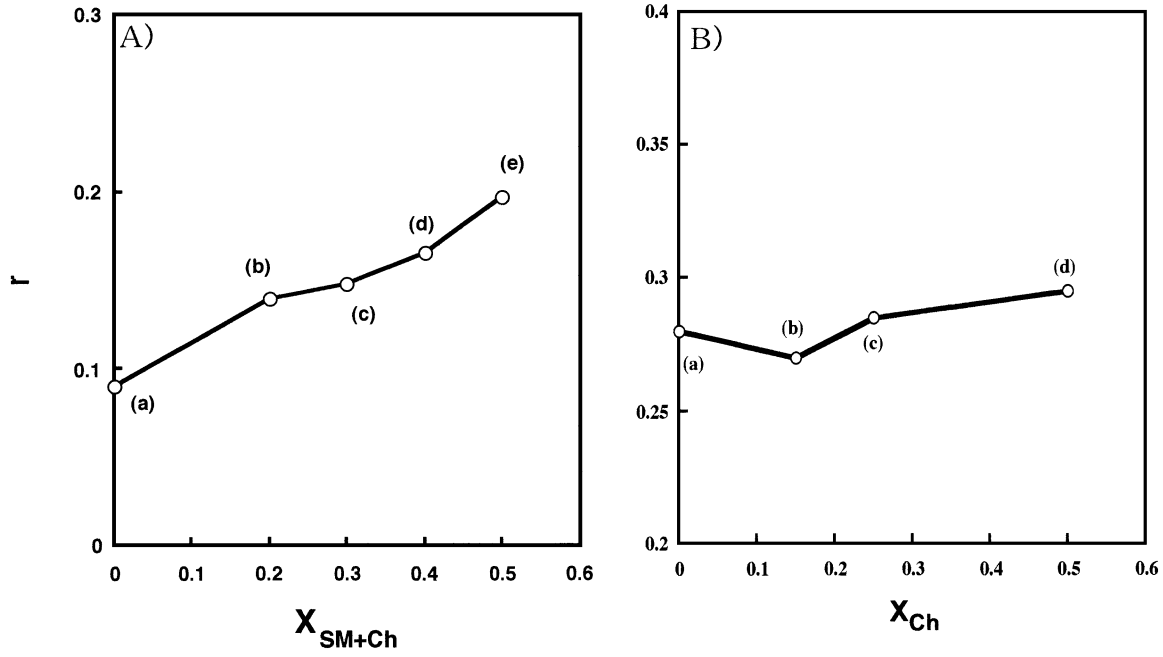

Fig. 6. Fluorescence anisotropy (r) of DHP in PC liposomes and PC–Ch–SM liposomes (A) and Ch–SM (B) composed of different phospholipid compositions. Excitation and emission wavelengths were 375 and 435 nm, respectively. Bandwidths (Ex/Em), 5 nm/5 nm were plotted. (A) Egg PC 100 μM (a); egg PC–SM–CH (80:10:10) (b); (70:15:15) (c); (60:20:20) (d); and (40:30:30) (e). (B) Brain SM 100 μM (a); Ch–SM (15:85) (b); (25:75) (c); and (50:50) (d).

same as that in PC–Ch (7/3). These indicate that the co-existence of SM and Ch in PC liposomes, that is, the raft of SM and Ch, promotes the association of the α-helical transmembrane peptides in lipid bilayers. These phenomena become much remarkable in the mixture system of SM–Ch (Fig. 5d). The fluorescence intensity of excimer in Ch concentration of less than 15 mol% was not so high, but in that of more than 25 mol%, it becomes the highest among all the lipid systems investigated here.

To investigate the aggregation factor of transmembrane peptides in the co-existence of SM and Ch in egg PC liposomes, we measured the membrane fluidity. A fluorescence probe, DPH, is generally employed for the elucidation of the order and dynamics of various membrane systems. Fig. 6 shows the fluorescence anisotropy of DPH (r) in egg PC liposome including SM and Ch and in binary mixture of SM and Ch under the same conditions as described in the aggregation experiments using excimer fluorescence of

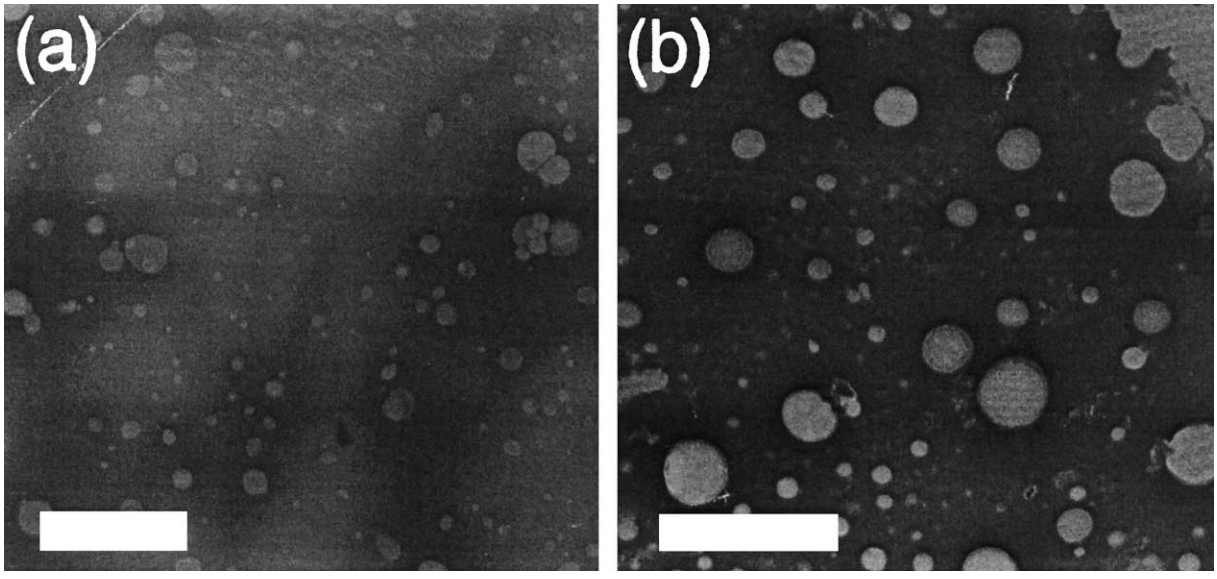

Fig. 7. Electron micrographs of negative-stained PC liposome (a) and PC–SM–Ch mixed liposome (4/3/3) (b) containing P_{24}Pya. Bar is 500 nm.

P_{24}Pya. With increasing the mixing ratios of SM and Ch against PC liposome, fluorescence anisotropies of DPH were increased almost linearly, suggesting that rotational motion of DPH in egg PC was restricted by the increasing concentrations of SM and Ch (Fig. 6a). These indicate that the mixture of SM and Ch, so-called the raft, decreases membrane fluidity and/or disturbs the lipid order with their increasing concentrations in phospholipid bilayer. Interestingly in binary mixture of SM and Ch, the fluorescence anisotropy firstly decreased up to 15 mol% and then increased with the increasing Ch concentrations (Fig. 6B). These may reflect the gel-to-liquid ordered phase transition of SM or SM–Ch bilayers by the increasing Ch as described below.

3.5. Electron micrograph study of mixed liposomes containing a transmembrane peptide

It is well known that the film of PC can make liposomes easily by hydration and sonication, but it is not clear whether that of PC including SM and/or Ch, and transmembrane peptides can make liposome. Thus, to ascertain whether mixed lipids containing a transmembrane peptide (P_{24}Pya) can make liposomes, we viewed the liposomes prepared by sonication as described in the Materials and Methods section. Fig. 7 shows electron micrographs of negative-stained PC liposomes and PC/–SM–Ch mixed liposomes (4/3/3) containing P_{24}Pya, respectively. The spherical liposomes (about 100 nm in diameter) are observed in Fig. 7, showing that both mixture systems can make liposomes even though P_{24}Pya is incorporated into lipid bilayers.

4. Discussion

Recent mathematical studies of membrane proteins indicate that there are some specific features on amino acid side chain distribution in transmembrane peptide segments. For instance, aliphatic residues (Leu, Ile and Val) are located largely in the fatty acyl chains of lipids and aromatic and Gly residues are enriched to the buried face (Ref. [1] as a review). The differential distributions of transmembrane side chains are likely to be the consequence of a few factors, such as hydrogen bonding, charge interaction within protein and the relative qualities of side chain/lipid and side chain/side chain packing. Thus, helix–lipid interaction and helix–helix interaction studies using the model transmembrane peptides can contribute to understand the fundamental factors of the folding of membrane proteins.

Hydrogen bond, electrostatic and dipole–dipole interactions are considered as association factors between α-helical transmembrane segments of membrane proteins [2]. We noticed that the electrostatic interaction of ion pairs could bring about the association of local regions of a pair of helices. Then van der Waals interactions would promote the detailed close packing and further stabilize the association. The energy transfer experiments using P_{24}EW (1 μM) and P_{24}Pya (1–8 μM) in the presence of PC liposomes (100 μM) indicated that transmembrane peptides are present closely (within 25 Å) in lipid bilayers under the present experimental conditions (Fig. 3). However, the difference of energy transfer effect between P_{24}EW–P_{24}Pya and P_{24}EW–P_{24}KPya was not observed even under the increasing concentration of ion pair peptides into lipid bilayers (Fig. 4), indicating no contribution of the charge interactions to the interhelical association. This raises the question of why the charge interaction did not contribute to the association of P_{24}EW–P_{24}KPya. There may be two reasons. One is that the charge interaction between transmembrane segments in lipid bilayer seems implausible, because of no penetration of charged group into non-polar region in lipid bilayers. This may not be the case because Lew et al. [23] have shown that an α-helical transmembrane peptide of 24-mer residues, K_2GL_7-(D or K)-LWL_9K_2A, with the highly hydrophobic α-helical transmembrane segment similarly to P_{24} is able to penetrate the segment containing charged residue into lipid bilayers at neutral pH. Monné et al. [24] have also shown that a model transmembrane peptide composed of 23 leucine residues and one valine residue with one positively or negatively charged residue can be also penetrated into lipid bilayers. Our present study also showed that P_{24}EW and P_{24}KPya are almost in the same conformation as P_{24}W (Fig. 2) and the peptides can make liposomes (Fig. 7), indicating that the strongly hydrophobic P_{24}EW and P_{24}KPya must also penetrate the hydrophobic segment containing charged residues into lipid bilayers.

The second explanation is that ionizable residues of Glu and Lys in the hydrophobic core of the bilayers exist in their uncharged states. There are several studies showing that in neutral pH, carboxy group in Asp residue has a p*K*a shifted up to 7 at the membrane surface [23,25]. No charged ω-amino group of Lys residue may also present at 6–8 pH units below its p*K*a values in solution [23]. The uncharged state(s) of one or both ionizable residue(s) may not lead to salt bridge formation in hydrophobic core of membrane, even though the chargeable residues are present in the hydrophobic core within transmembrane α-helical segments. These may suggest that charge interaction in lipid bilayers is not so important to promote or keep the helix–helix association, that is, the folding structure of integral membrane protein. But we do not want to overestimate the inability of salt bridge in multiple-spanning membrane proteins. It is considered that membrane proteins are "inside out" as compared with soluble proteins; their outside surface is more hydrophobic than the protein core. Charged residues would be able to stabilize the helix–helix bundle by salt bridge at more hydrophilic inside protein core in membrane proteins.

As transmembrane α–helical segment is highly hydrophobic, the membrane lipids can promote membrane protein folding as a solvent. But it is not clear as to whether lipids participate to promote and maintain the helix–helix associ-

ation. According to the transmembrane peptide aggregation experiments using the excimer formation of P_{24}Pya, when Ch contents in egg PC–Ch mixture were increased by 20 mol% and by 30 mol%, the excimer fluorescence due to P_{24}Pya was observed. The intensity of 30 mol% Ch was much stronger than that of 20 mol%, meaning that the peptide aggregations were increased much largely in 30 mol% Ch (Fig. 5b). Mixtures of SM or DPPC and Ch are known to form two liquid-crystalline phases, liquid ordered (Lo) phase and disordered phase (Ld), depending on lipid compositions. The phase diagrams for binary mixture of Ch with PC show that Ld phase and Lo phase exist in the Ch concentration range 0 to 7–23 mol% and 25–33 to 50 mol%, respectively [26,27]. In intermediate concentration range, the Ld and Lo coexist. Thus, the dependence of peptide aggregations in egg PC–Ch mixture on the Ch contents may come from the difference of the membrane fluidity due to phase separation between two liquid-crystalline phase as described below.

When the content ratios of SM and Ch were increased in the egg PC liposomes, the fluorescence intensities of excimer, particularly in PC–Ch–SM (4:3:3) liposome, were much stronger than those of the corresponding Ch contents in the mixed bilayers of egg PC–Ch (Fig. 5c), indicating that the peptide associations were more promoted. SM and Ch are also known to form the Lo phase [28]. The phase separation observed in the Ch-containing egg PC–SM membranes has been correlated to the membrane fluidity [29]. The decreasing membrane fluidity by increasing the Lo phase may be the reason why the complex of SM and Ch contributes to peptide aggregation in lipid bilayers. Egg PC liposome is in the fluid state because fluorescence anisotropy of DHP in the liposomes had a low value, about 0.1 as shown in Fig. 6. The increasing contents of SM and Ch in egg PC/SM liposomes led to the decrease in the membrane fluidity, and the fluorescence anisotropy value in PC–Ch–SM (4:3:3) was about 0.2, meaning that the mixed liposomes are in coexistence between a Lo phase and liquid Ld phase [29]. We interpret that transmembrane α-helical peptide can move easily in the fluid membrane of egg PC liposomes. But in the mixture lipid membranes of SM–Ch–PC, the peptides are extruded from the Lo domains of the complex formed by SM and Ch to the Ld domain of PC. Since the increase in the contents of SM and Ch leads to the decreases in the content of Ld domain, the moving area of transmembrane peptide is limited in the liposomes. Thus, P_{24}Pya, transmembrane peptide, P_{24}Pya, can easily aggregate to form the excimer in the presence of the lipid-raft.

These are also supported by the excimer formation of P_{24}Pya in SM–Ch binary systems. The ability of excimer formation depends on the Ch concentration in SM (Fig. 5d). Intensity of excimer peak around 450 nm was not high in less concentration of Ch than 15 mol%, but it became remarkably higher in more than 25 mol% and the highest in 50 mol%. It is well characterized that brain SM transforms the phase from gel state to Lo phase with increasing Ch in SM bilayers [30,31]. Particularly, equimolar Ch and SM are completely Lo phase. Gel phase of SM and Lo domain of Ch and SM coexist in 0<Ch<50 mol%. In our present study, the excimer fluorescence in only SM bilayer is very low, indicating that the moving of pyrene chromophore in the peptide is restricted in gel state, but with increasing Ch, the phase separation between peptide and lipid in Lo domain lead to the easy aggregation of P_{24}, resulting in the excimer formation. The strongest excimer formations in equimolar Ch and SM may come from the close aggregation by no Ld domain in Ch–SM bilayers. Large difference of excimer formation ability between 15 and 25 mol% Ch in SM may be attributable to the decrease of fluorescence anisotropy by addition of Ch to SM bilayer (Fig. 6b). In this connection, transition enthalpy (ΔH) is drastically decreased up to 15 mol% Ch, then gradually and finally completely disappeared at 50 mol% Ch for hydrated brain SM including Ch on differential scanning calorimetry experience [30].

We could not exclude the possibility that the complex of Ch and SM includes the transmembrane peptides into Lo phase (the raft). However, this possibility is very low because the excimer formation of peptides in lipid bilayers depends on the concentrations of SM and Ch; if the peptides were dissolved into the raft, the increasing concentration of SM and Ch would lead to decrease in the excimer formation.

The present study using model membranes showed the evidence that the complex of SM and Ch contributes to the aggregation of transmembrane peptides. But we could not correlate the results obtained to a role of rafts in plasma membranes; polarized protein sorting and signal transduction. It should be continuously investigated whether peptide-linked rafts in lipid bilayer are present or not, using more practical biomembrane.

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