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Fusion of dioleoylphosphatidylcholine vesicles induced by an amphiphilic cationic peptide and oligophosphates at neutral pH

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Peptide E5 is an analogue of the fusion peptide of influenza virus hemagglutinin and K5 is a cationic peptide which has an arrangement of electric charges complementary to that of E5. We reported that a stoichiometric mixture of E5 and K5 caused fusion of large unilamellar vesicles (LUV) of neutral phospholipids (Murata, M., Kagiwada, S., Takahashi, S. and Ohnishi, S. (1991) J. Biol. Chem. 266, 14353–14358). K5 caused fusion of LUV composed of dioleoylphosphatidylcholine (DOPC) at pH > 10, but not at neutral pH. In the presence of oligophosphates, such as 1 mM ATP, GTP, or polyphosphate, K5 caused rapid and efficient fusion of DOPC LUV at neutral pH without hydrolysis of oligophosphate groups, but another anions such as citrate, acetate, AMP, phosphate, or EDTA were ineffective. The peptide/oligophosphate-induced fusion behaviors have been investigated by a fluorescence resonance energy transfer assay for lipid mixing of LUV and negative staining electron microscopy. At higher ionic strengths (> 0.3 M KCl) or in the presence of 5.0 mM $MgCl_2$, the fusion was inhibited. Even at the inhibitory conditions, the association of K5 with lipid vesicles at neutral pH was directly confirmed by the Ficoll gradient assay method and by blue shifts of the tryptophan fluorescence of the peptide. A nonhydrolyzable GTP analogue, GTPγS, also induced fusion. These observations suggested that the electrostatic interactions between the positive and negative charges of K5 and oligophosphate, respectively, induced complex formation, triggering membrane fusion.

Introduction

Membrane fusion plays a crucial role in intracellular vesicular transports, intercellular fusion and viral infectivity. The fusion might be mediated by a specific protein (or protein complexes) and controlled strictly by molecules of low molecular weights, such as Ca^{2+}, H^+, or ATP [1–3]. H^+-induced membrane fusion mediated by protein has been extensively studied, especially for the viral fusogenic proteins, HA of influenza virus, G protein of vesicular stomatitis virus and E1 protein of Semliki Forest virus. They are the main envelope proteins of these viruses and cause low-pH-induced fusion between their viral envelope and the endosomal membranes of a target cell to transfer the viral genomes into the cytoplasm. It has been supposed for HA that the low pH in endosomes induced a conformational change in the protein to expose a 'fusion peptide', which is the conservative N-terminal region of HA-2 subunit. Positive evidence of a participation of fusion peptides in virus-cell fusion has been discussed [2,3]. The synthetic peptides, having the same amino-acid sequence as the fusion peptide of HA, caused pH-dependent or -independent fusion of SUV composed of neutral phospholipids [4–6]. Also, a peptide of 30 amino-acid residues, as an analog of fusion peptides, has been shown to induce fusion of phosphatidylcholine SUV at acidic pH values, upon formation of a helical structure [7]. A 23-residue synthetic peptide which was related to an arenavirus protein was also reported to induce pH-dependent lipid mixing of SUV [8].

Recently, we have synthesized several amphiphilic peptides and studied the relationship between their secondary structures and the membrane fusion activity [9]. The anionic peptides, as analogs of the influenza

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Abbreviations: AMP-PNP, adenosine 5′-[β,γ-imido]-triphosphate; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DOPC, dioleoyl-L-α-phosphatidylcholine; DPX, *p*-xylene-bis-pyridinium bromide; GTPγS, guanosine 5′-[γ-thio]triphosphate; HA, hemagglutinin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LUV, large unilamellar vesicles; NBD-PE, dipalmitoyl-L-α-phosphatidyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethanolamine; R_{18}, octadecylrhodamine B; SUV, small unilamellar vesicles.

E5 H_2N-G L F E A I A E F I E G G W E G L I E G-CO_2H
K5 H_2N-G L F K A I A K F I K G G W K G L I K G-CO_2H

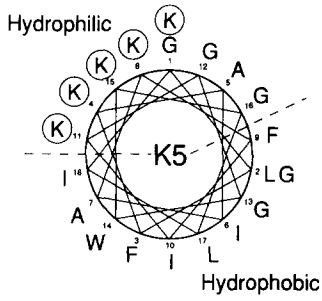

Fig. 1. The primary structure of K5 and E5 and a helical wheel representation of K5.

HA fusion peptide, caused fusion of DOPC LUV at acidic pH and the cationic one at alkaline pH, although both of them showed no fusion at neutral pH. A mixture of the anionic peptide and the charge-reversed cationic peptide, however, induced the fusion at neutral pH [9]. Taken together, we propose that the charge-neutralized forms of the peptides are more hydrophobic than those having electric charges and can interact with neutral phospholipid bilayers and aggregate the apposed vesicles to cause fusion. In these cases, the charge neutralization was achieved (i) for anionic peptides by the protonation of acidic residues; (ii) for cationic peptides by the deprotonation of the basic residues; (iii) or upon neutralization of electric charges by a mutual interaction between peptides having complementarily arranged electric charges to each other.

In this paper, we report that the cationic peptide K5 (Fig. 1) induces the fusion of DOPC LUV at neutral pH in the presence of low molecular weight oligophosphates, such as ATP, GTP and polyphosphate. The fusion is triggered by electrostatic and sterically specific interaction of the negative charges of an oligophosphate with the positive charges of K5. This is another example of the third type of fusion described above and demonstrates a presence of peptide-induced membrane fusion regulated by a participation of low molecular weight molecules.

Materials and Methods

Materials

Peptide K5 was synthesized as described [9]. Concentrations of K5 were determined from the absorbance at 280 nm using the molar extinction coefficient 5500 $M^{-1}cm^{-1}$ at pH 7.0–8.5. DOPC was purchased from Sigma and phospholipid concentrations were determined by the Bartlett method [11]. Fluorescence probes, ANTS, DPX, NBD-PE, and R18 were purchased from Molecular Probes. Ficoll and polyphosphate (degree of polymerization approx. 4) were obtained from Nacalai Tesque (Kyoto). Sialic acid and all nucleotides were from Sigma. The pH values of all the anion solutions we tested were adjusted to 7.4 with NaOH.

Assay of vesicle fusion and leakage

Aqueous contents mixing of DOPC LUV upon fusion was measured by the ANTS/DPX method as described by Ellens et al. [12]. DOPC LUV were prepared by the reverse-phase evaporation method [13] and contained either (i) 25 mM ANTS, 40 mM NaCl, 10 mM Tris-HCl (pH 7.5) or (ii) 90 mM DPX, 10 mM Tris-HCl (pH 7.5). LUV were extruded through 0.2-μm and 0.1-μm polycarbonate filters (Nuclepore) successively and separated from uncapsulated materials by chromatography on Sephadex G-75 with 100 mM NaCl, 10 mM Hepes-NaOH (pH 7.5). For fusion assay, an equimolar mixture of ANTS- and DPX-vesicles was suspended in 0.3 ml of Hepes buffer (5 mM Hepes, 145 mM KCl, final lipid concentration was 1.1 mM). At $t = 0$, a small volume of peptide K5 solution was added to the vesicle suspension at a lipid to peptide molar ratio about 40. A decrease in ANTS fluorescence at 530 nm upon fusion was monitored at room temperature with the excitation wavelength at 384 nm. A low-cut filter (Fuji Film, SC500) was placed in the emission optical path to reduce scattering contribution. 0% fusion was the fluorescence intensity of an LUV suspension before an addition of the peptide. The 0% fluorescence level was taken as 100% fusion.

Leakage of vesicle contents was assayed independently by using LUV which contained 12.5 mM ANTS, 45 mM DPX and 10 mM Tris-HCl (pH 7.5). The fluorescence intensity of the LUV suspension after an addition of Triton X-100 (the final concentration of the detergent was 0.3%) was taken as the level of 100% leakage. Usually, we added the peptide to vesicle suspensions (0.64 mM) at pH 7.5 containing one of anions to be tested. For another experiment, we added the peptide to the vesicle suspension at neutral pH and incubated for 1 min, and then added to the mixture an anion which should be tested.

Lipid mixing of vesicles upon fusion was assayed with fluorescent probes as described by Struck et al. [14]. DOPC LUV containing 1 mol% each of NBD-PE and R_{18} were prepared in Hepes buffer (145 mM KCl, 5 mM Hepes (pH 7.4)) with final lipid concentration of 1.1 mM. An equimolar (or 1:4) mixture of the labeled and unlabeled vesicles was suspended in 0.3 ml of this buffer and the pH was adjusted to a desired value. At $t = 0$, a small volume of the peptide solution was added to the vesicle suspension. An increase in NBD fluorescence at 530 nm upon fusion was monitored with the excitation at 450 nm. The fluorescence intensity of LUV suspension after an addition of Triton X-100 (the

final concentration was 0.3%) and that before an addition of the peptide was assumed as the level of 100% and 0% fusion, respectively. Inorganic phosphate was quantitated as described by Nathan et al. [15].

Fusion of vesicles was also investigated by negative staining electron microscopy. DOPC LUV were incubated in the presence of K5 and various oligophosphates (1.0 mM), stained with 3% uranyl acetate, and viewed through a JEOL 100X electron microscope.

Physicochemical characterization of K5

Binding of K5 to DOPC vesicles was assayed by flotation centrifugation with Ficoll as described by Shen et al. [16]. A suspension (0.1 ml) of DOPC vesicles containing fluorescent marker NBD-PE (1% of total lipids) was mixed with 50 μl of K5 solution (8–180 μM) and 150 μl of the Hepes buffer containing 1 mM ATP and incubated at room temperature (23°C) for 10 min. For the other experiment, after preincubation of K5 with 1 mM ATP at room temperature for 2 min, the mixture was added to DOPC vesicles and incubated for 10 min as for binding assay. The suspension was mixed with 300 μl of 25% Ficoll in the Hepes buffer. 2 ml of 10% Ficoll in the same buffer and the buffer itself were overlaid to the suspension sequentially and centrifuged at $2000 \times g$ for 30 min. After centrifugation, fractions of 50 μl each were taken from the bottom to the top. The peptide and lipid contents of each fraction were determined by the fluorescamine method and NBD fluorescence, respectively.

The fluorescence spectrum of the tryptophyl residue was measured with the excitation at 285 nm for the peptide at 21 μM in the presence or absence of DOPC LUV (0.65 mM). The 90° light scattering of vesicle suspensions was measured at 400 nm to obtain the information on aggregation and fusion of vesicles.

The CD spectra were measured with a JASCO J-20 spectropolarimeter modified with a quartz stress modulator and an Ithaco lock-in amplifier. When a nucleotide was included in a sample, the contribution of the nucleotide was subtracted from the spectrum of a mixture. Ellipticity is expressed in mean residue weight basis.

Results

Fusion induced by K5 in the presence of various oligophosphates at neutral pH

The influence of various monovalent or multivalent anions on K5 for fusion of DOPC LUV at neutral pH was investigated by lipid and contents mixing assay. Time-courses of lipid mixing (detected as a fluorescence resonance energy transfer between NBD-PE and R_{18}) caused by K5 and a various kind of anions are shown in Fig. 2. When K5 was added at $t = 0$ to a 1:4 mixture of the labeled and nonlabeled LUV, a small amount of lipid mixing at neutral pH, an intrinsic effect of K5, was observed. The gradual increase of lipid mixing was not accompanied with contents mixing of LUV under the conditions, suggesting that it was not due to the fusion but due to the aggregation of vesicles (see Ref. 9). 1 min later an anion under consideration was added to the mixture of K5 and LUV at 1–10 mM concentration. Ascorbic, sialic, phosphoric, acetic, citric, ethylenediaminetetraacetic and propane-1,2,3-tricarboxylic acids did not enhance the lipid mixing at neutral pH and showed a gradual increase in lipid mixing at the same rate observed for K5 (Fig. 2, curve c). On the contrary, ATP or ADP induced rapid and efficient fusion of DOPC LUV at a threshold concentration about 0.1 mM (Fig. 2, curves a and b). The effect of ATP on the fusion was larger than that of ADP; the effect of 1.5 mM ADP was comparable with that of 1 mM ATP (Fig. 2, curve d). In contrast, AMP did not induce the lipid mixing at the concentrations ranging from 1 to 10 mM (Fig. 2, curve c). The fusion efficiency was independent of the order of an addition of K5 and ATP to the LUV suspension (see Fig. 6).

We compared the effects of other nucleoside triphosphates other than ATP on K5-mediated fusion of DOPC LUV when the nucleotide concentration held constant at 1.0 mM. Labeled lipid vesicles were mixed with nonlabeled vesicles at a molar ratio 1:4 for the lipid mixing assay, which showed that the K5-mediated fusion was dependent on the kind of nucleotides with the following order of effectiveness: GTP $\approx$ ATP $\gg$ UTP $\approx$ CTP (Fig. 3A). The initial rate and the extent of fusion were also dependent on a basic group of nucleotides, those induced by purine nucleotides were higher than those by pyrimidine nucleotides. The

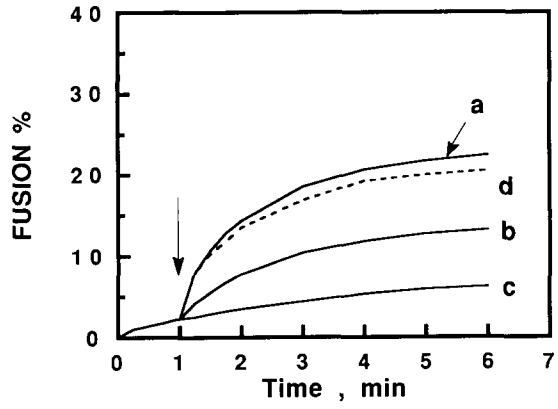

Fig. 2. A typical time-course of fusion of DOPC LUV induced by K5 at neutral pH. The fusion was monitored by lipid mixing assay described in Materials and Methods. To a 1:4 (molar ratio) mixture of labeled and nonlabeled LUV (total 1.0 mM), K5 (33 μM) was added at room temperature and at $t = 0$ and then various anions were added at time 1 min later (indicated by an arrow). Curve (a), 1.0 mM ATP; (b), 1.0 mM ADP; (c), 1.0 mM AMP or other anions described in the text and (d), 1.5 mM ADP.

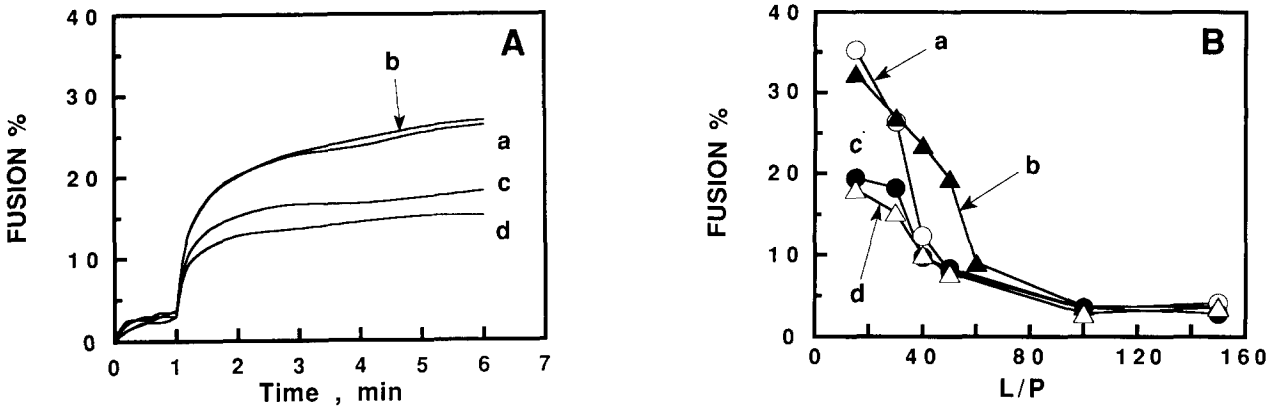

Fig. 3. Nucleoside triphosphate-dependent fusion of DOPC LUV induced by K5. (A) Time-course of the fusion induced by various nucleoside triphosphates. The fusion was monitored by lipid mixing assay described in Materials and Methods. To a 1:4 mixture of labeled and nonlabeled LUV (1.0 mM), K5 (33 μM) was added at room temperature at time 0 and then various nucleoside triphosphates (1.0 mM) were added 1 min later. Curve (a), ATP; (b), GTP; (c), CTP and (d), UTP. (B) Dose-dependence of fusion induced by various nucleoside triphosphates. The extent of fusion by lipid mixing assay was measured at 3 min after the addition of nucleoside triphosphates (1.0 mM) and plotted against the lipid-peptide ratio (L/P). The vesicle concentrations were held constant at 1.0 mM. (a), ATP; (b), GTP; (c), CTP and (d), UTP.

extent of lipid mixing was also dependent on lipid/peptide ratio (Fig. 3B). When the ratio was higher than 70, lipid mixing was not observed in any case. Polyphosphate (mean degree of polymerization approx. 4) caused the most efficient fusion among the oligophosphates so far studied with our assay methods (Fig. 7B). We found that there was no liberation of P_i from nucleoside triphosphates and polyphosphate during these experiments.

Fusion was also assayed by the internal contents mixing of ANTS-containing LUV and DPX-containing LUV. For the contents mixing assay, anions were added to the LUV suspension at first and 1 min later K5 was added, because K5 caused a little leakage of DOPC LUV without fusion at neutral pH, which would interfere the assay [10]. Even with that caution, leaking of a lot of the contents from vesicles was observed (Fig. 9A,B). Consequently, we could not determine mixing of the internal contents of liposomes on fusion. No definite conclusion whether the leakage occurred during vesicle fusion (leakage at the point of fusion) or was not associated with the fusion process (independent leakage), has not been obtained yet.

The 90° light scattering of a mixture of K5 and LUV slightly increased as compared with the level of LUV themselves, indicating that K5 caused a little aggregation of vesicles under the conditions. When sialate, acetate, citrate, phosphate, or EDTA was added to the mixture of K5 (26 μM) and LUV (0.8 mM), the scattering was nearly the same as that of the mixture of K5 and LUV. This suggested that aggregations of LUV were not enhanced in the presence of one of these anions. However, when the ATP or ADP was added to the mixture, the scattering increased proportionally to the nucleotide concentration in a range of 0.1–1.0 mM (data not shown), suggesting that the fusion or aggregation of DOPC LUV was induced by the nucleotides.

Interestingly, at a low lipid/peptide ratio, there was a transient, rapid increase in light scattering which rapidly decreased and then gradually increased. Such

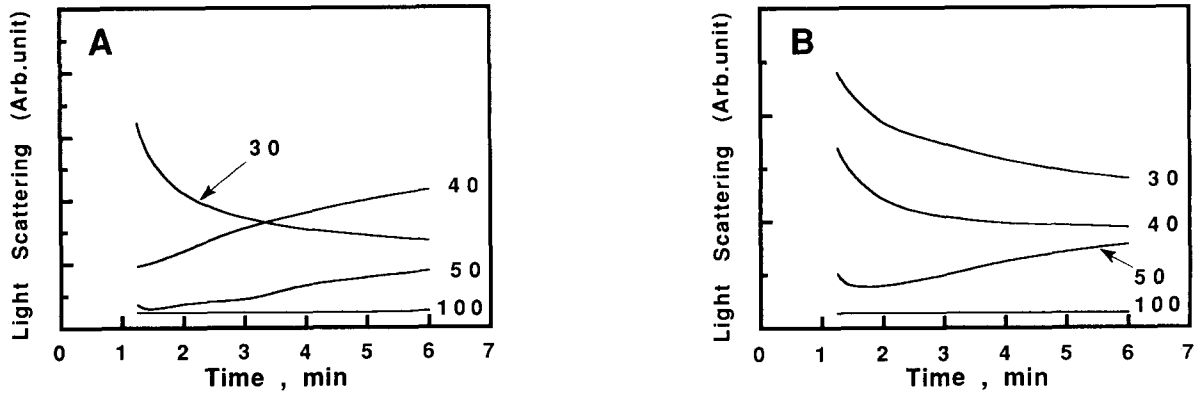

Fig. 4. Light-scattering measurements. 1.0 mM ATP (A), or 1.0 mM GTP (B) was added to the mixture of DOPC LUV (1.0 mM) and K5 (33 μM) at $t = 0$. Lipid/peptide ratio (L/P) was as indicated in the figures.

kinetics of light scattering were only observed at low lipid/peptide ratios, for example at lipid/peptide < 30 for the K5–ATP system, or at lipid/peptide < 50 for the K5–GTP system. This suggested that large, transient aggregation of vesicles might occur in the early step of the fusion (Fig. 4).

Fusion was also investigated with negative staining electron microscopy. When LUV (1.0 mM) were incubated with K5 (25–33 μM) at pH 7.4, vesicles remained small with diameters of 0.1–0.3 μm. These diameters were consistent with our results of the dynamic light-scattering measurements for LUV (d = 193 ± 1.23 nm). On incubation of LUV (1.0 mM) with K5 (25–33 μM) and 1.0 mM ATP (or GTP) at pH 7.4 for 30 s, many large aggregates of vesicles were observed at lipid/peptide = 30 for K5–ATP system (Fig. 5B,D), or at lipid/peptide = 40 for K5–GTP system as expected from the light-scattering profiles (Fig. 4). On incubation of the samples for 5 min, the larger aggregates disappeared and the enlargement of some vesicles was observed with diameter of 1–2 μm (Fig. 5C,E). The light scattering of the mixture of K5 and

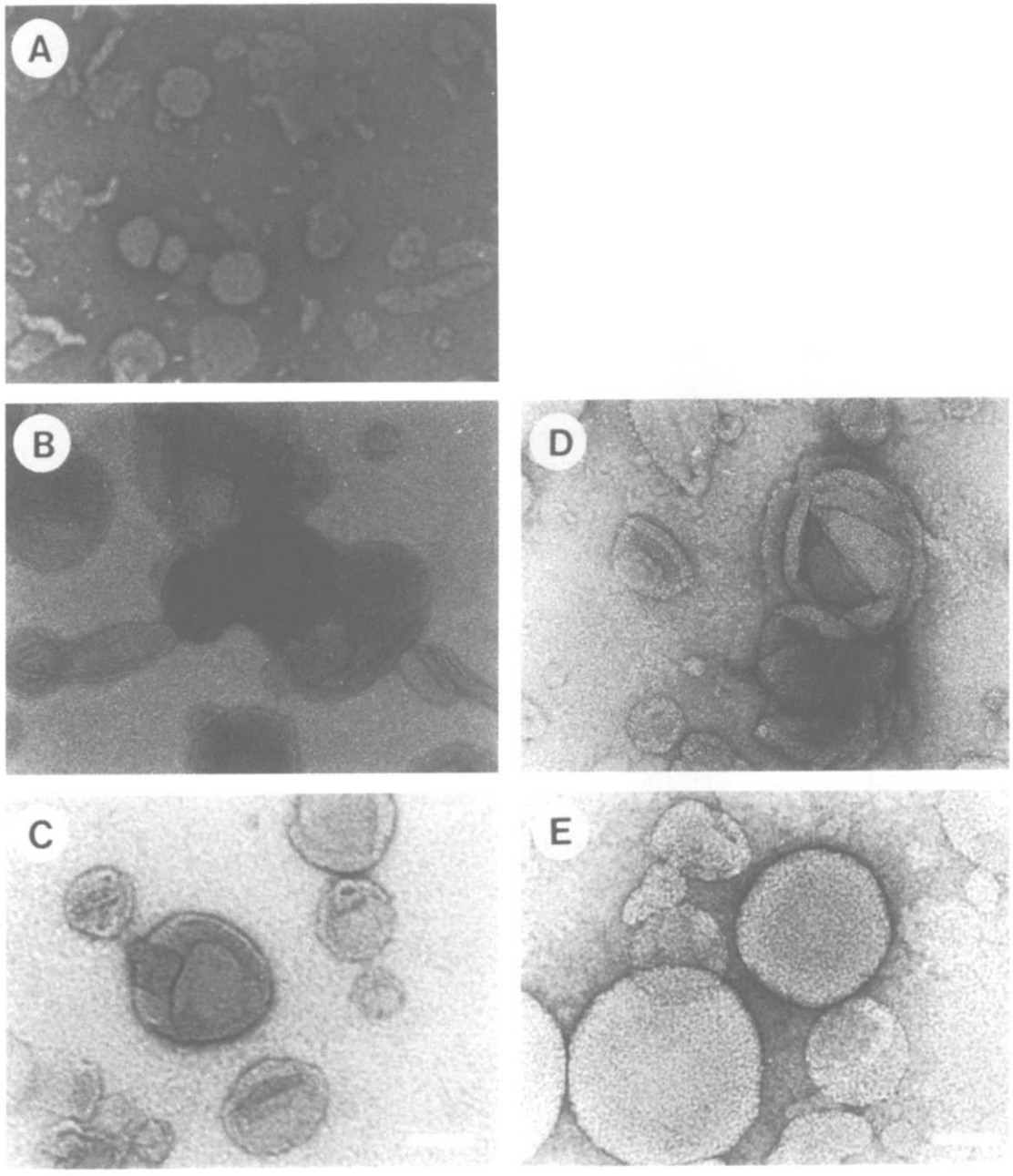

Fig. 5. Electron micrographs of DOPC LUV (1.0 mM) incubated with K5 and 1.0 mM ATP at pH 7.4 for the time indicated in the figures at room temperature. Lipid-peptide ratio (L/P) was 30 for A, B and D or 40 for C and E. (A) K5 + LUV with 5 min incubation; (B,C) K5 + LUV + 1.0 mM ATP with 30 s incubation; (D,E) K5 + LUV + 1.0 mM ATP with 5 min incubation.

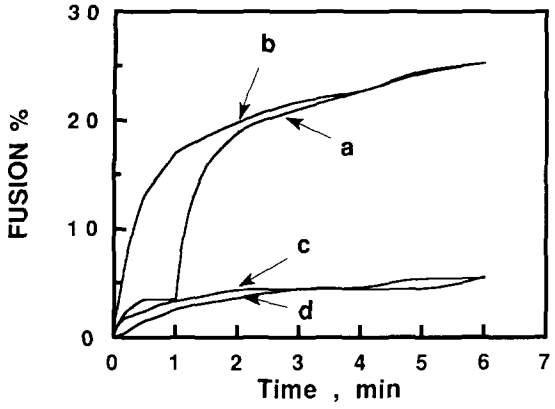

Fig. 6. ATP-dependent fusion of DOPC LUV induced by K5. The fusion was assayed by lipid mixing, described in the text. (a) Fusion was initiated by the subsequent addition of K5 (33 μM) and then 1.0 mM ATP (1 min later), or (b) by the subsequent addition of 1.0 mM ATP and then K5 (33 μM, at $t = 0$). (c) Fusion was initiated at $t = 0$ by the addition of a 2-min-preincubated mixture of K5 (33 μM) and 1.0 mM ATP, or (d) by the addition of K5 (33 μM) only.

nucleotide (or polyphosphate) is negligible under these experimental conditions.

Decrease in fusion efficiency by preincubation of the peptide with ATP

In fusion assays, we added K5 and ATP successively to LUV suspensions (Fig. 6, curve a). Preincubation of a mixture of K5 and ATP (1.0 mM) for 2 min at room temperature before the addition to LUV suspensions caused a drastic decrease in the extent of fusion (Fig. 6, curve c). In this experiment, we were afraid that precipitation of the complex of K5 and ATP from the mixture might reduce the extent of fusion by reducing the degree of association of the peptide with lipid vesicles. We, therefore, studied the degree of association of preincubated K5 and lipid vesicles by the Ficoll floating assay. In the absence of vesicles, K5 remained at the bottom fractions of the Ficoll gradient. When K5 (26 μM) and 1.0 mM ATP were added to a vesicle suspension (lipid concentration 0.8 mM) successively and the mixture was kept for 5 min at pH 7.4 (lipid to peptide ratio was 30) before being applied to the Ficoll gradient, K5 comigrated with vesicles after the centrifugation. Preliminary incubation of a mixture of K5 (33 μM) and 1.0 mM ATP for 2 min at pH 7.4 prior to the addition of the mixture to a vesicle suspension did not change the result. We concluded that K5 preincubated with ATP did associate with vesicles to a similar extent as without preincubation.

Retardation of membrane fusion at high ionic strengths or in the presence of magnesium chloride

The nucleoside-triphosphate-dependent K5-mediated fusion was strongly sensitive to the ionic strength of the reaction mixture. At higher ionic strengths (> 0.3

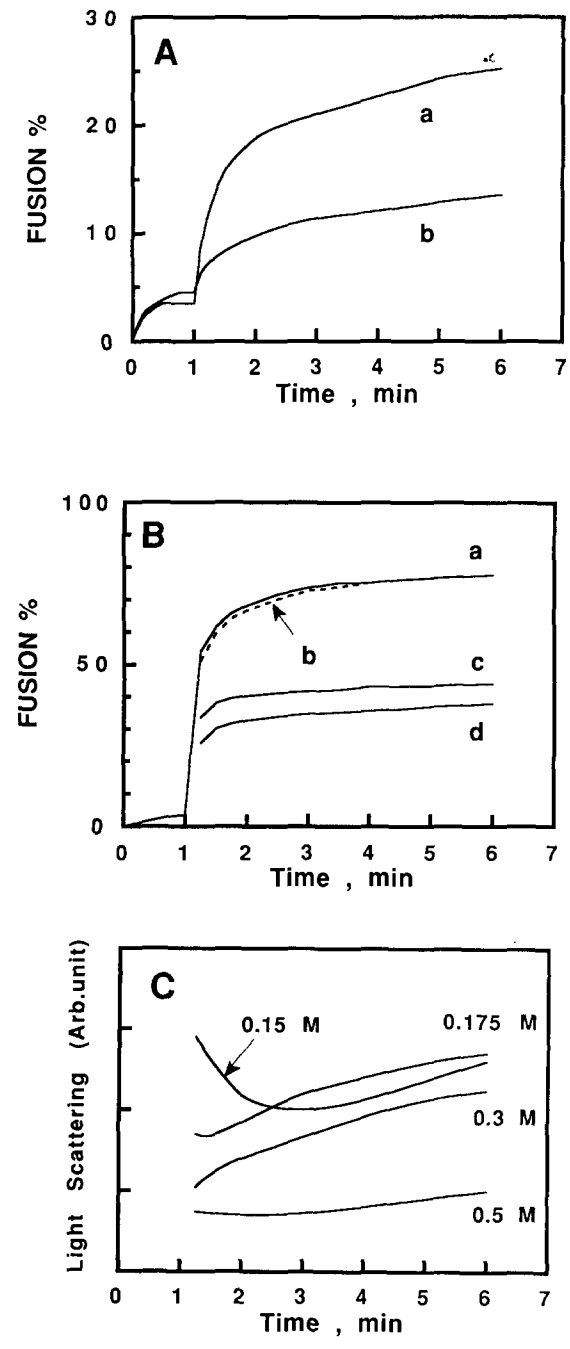

Fig. 7. Retardation of the fusion triggered by 1.0 mM ATP (A) at higher ionic strength, or of the fusion triggered by polyphosphate in the presence of $MgCl_2$ (B). (A) K5-induced fusion of DOPC LUV in the presence of 1.0 mM ATP in a low ionic strength buffer (a; 5 mM Hepes, 145 mM KCl (pH 7.4)), or in a high ionic strength buffer (b; 5 mM Hepes, 300 mM KCl (pH 7.4)). (B) K5-induced fusion of DOPC LUV in the presence of 1.0 mM polyphosphate in the low ionic strength buffer (a), in the high ionic strength buffer (b), in the presence of 5 mM (c) or 10 mM $MgCl_2$ (d) in the low ionic strength buffer. (C) Light-scattering measurements of the mixture of DOPC LUV and K5 in the presence of 1.0 mM GTP at variable ionic strength (5 mM Hepes, 0.15–0.5 M KCl (pH 7.4)). The concentration of KCl were indicated in the figure. For (A), (B) and (C), nucleoside triphosphates (A,C), or polyphosphate (B) was added to the mixture of DOPC LUV (1.0 mM) and K5 (33 μM) at 1 min. Fusion was monitored using the lipid mixing assay described in Materials and Methods.

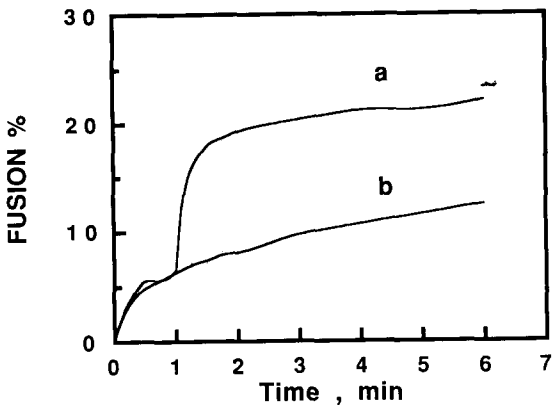

Fig. 8. Effect of the nonhydrolyzable nucleoside triphosphate (AMP-PNP) on K5-mediated fusion. The fusion was monitored using the lipid mixing assay described in Materials and Methods. To a mixture of DOPC LUV (1.0 mM) and K5 (33 μM), 1.0 mM ATP (a) or 1.0 mM AMP-PNP (b) were added at 1 min.

M KCl), the fusion was completely inhibited for all nucleoside triphosphates at the concentration of 1.0 mM (Fig. 7A). The light scattering of the LUV suspension was not changed under these conditions, indicating that no appreciable aggregation or fusion occurred. The polyphosphate-dependent K5-mediated fusion was insensitive to high concentrations (up to 1.0 M) of KCl (Fig. 7B, curve b), but sensitive to the presence of Mg^{2+} (Fig. 7B, curves c and d). At higher ionic strengths, the rapid and transient increase in light scattering during the early stage of the fusion at small lipid/peptide ratio was not observed (Fig. 7C).

Effect of nonhydrolyzable nucleoside triphosphate analogs on K5-induced fusion

We studied the effect of AMP-PNP and GTPγS, which were nonhydrolyzable analogs of ATP and GTP, respectively, on the K5-mediated fusion using the lipid mixing assay. AMP-PNP inhibited the fusion. Unlike the K5-ATP system, the fusion efficiency was dependent on the order of an addition of K5 or AMP-PNP to the LUV suspension (Fig. 8). When the LUV and K5 were preincubated and then AMP-PNP was added to the LUV suspension, the fusion was completely inhibited. When the LUV and AMP-PNP were preincubated, after which K5 was added to the LUV suspension, the inhibition was only partial (about 50% of the control). On the other hand, GTPγS (1.0 mM) did induce the K5-mediated fusion to the same extent as GTP.

Leakage induced by K5 in the presence of oligophosphates

Leakage of the fluorophore ANTS/DPX complex from LUV, induced by K5 or K5-oligophosphate, was determined by dequenching of the fluorescence of ANTS which was released into the medium. K5 itself (21 μM) caused leakage at neutral pH (possibly without fusion of vesicles as described previously), suggesting that K5 interacted with DOPC LUV under the conditions. The K5-induced leakage was facilitated to a great extent when oligophosphate was added to the mixture of K5 (21 μM) and LUV (0.64 mM) (Fig. 9). The fusion-inducible ATP or ADP enhanced the leakage to a great extent but AMP or other anions studied so far did not.

As shown in Fig. 9B, the oligophosphates themselves did not cause the leakage. When K5 (21 μM) was added to a mixture of oligophosphate (1 mM) and LUV (0.64 mM) 1 min after the mixture was prepared, an extensive leak was induced to the same extent as when the peptide and the oligophosphate were added in this order to the LUV suspension (Fig. 9A,B). A preincubated mixture of K5 and 1.0 mM ATP also caused leakage under similar conditions but the initial

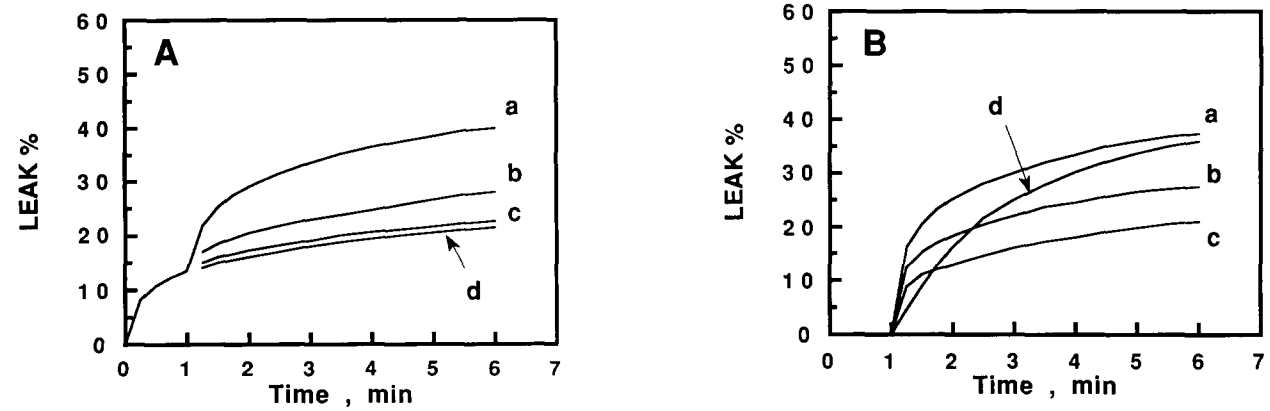

Fig. 9. Leakage of DOPC LUV induced by K5 in the various conditions. (A) To ANTS/DPX-containing LUV (0.64 mM), K5 (21 μM), was added at $t = 0$ and 1 min later the nucleotide studied (1.0 mM) was added. Curves (a) ATP, (b) ADP, (c) AMP and (d) without any nucleotides. (B) To ANTS/DPX-containing LUV (0.64 mM), various anions (1.0 mM) were added at $t = 0$ and then K5 (21 μM) was added 1 min later. Curves (a) ATP, (b) ADP, (c) AMP and (d) preincubated mixture of K5 and ATP. Leak % was estimated by the method described in Materials and Methods.

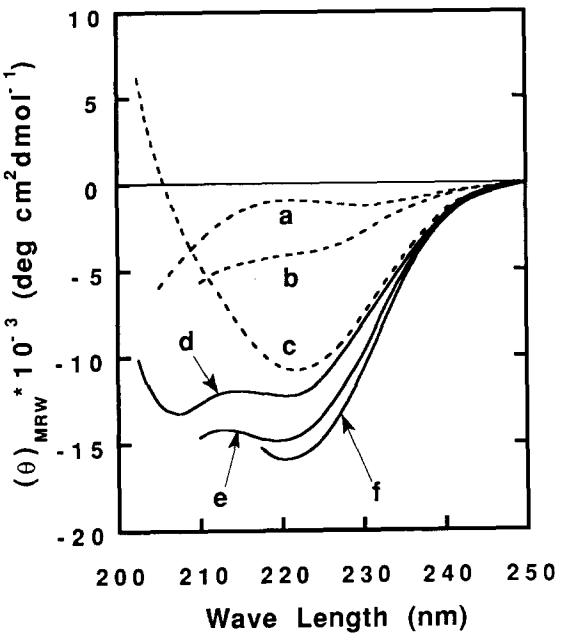

Fig. 10. CD spectra of K5. K5 concentration was $1 \cdot 10^{-5}$ M. Concentration of oligophosphates were $3 \cdot 10^{-4}$ M, except for (f). (a,b,c) In the absence of lipid. (a) K5 in 5 mM Hepes, 0.15 M KCl (pH 7.4); (b) +ATP; (c) +polyphosphate. (d,e,f) In the presence of 0.3 mM DOPC LUV. (d) K5; (e) +ATP or GTP; (f) +polyphosphate ($3 \cdot 10^{-5}$ M).

rate of leakage was smaller than that observed for the successive additions (Fig. 9B, curves a and d), suggesting that the preincubated mixture, which was ineffective to the fusion, interacted with vesicles and disturbed vesicle integrity.

CD studies

The conformation of K5 at neutral pH is unordered or α-helical in the absence or presence of lipid bilayers, respectively [9]. When a nucleotide was added to K5, precipitates or aggregates were observed if K5 concentration was higher than 15 μM (0.3 mM nucleotide or polyphosphate). Therefore, CD measurements were restricted to solutions with K5 concentrations lower than 10 μM. In the absence of lipid bilayers, an appreciable increase in the ellipticity near 220 nm was observed when ATP was added to a K5 solution at neutral pH (Fig. 10). Although the estimated amount of ordered structure (any of α-helix or β-structure) was less than 30% [17,18], a triphosphate-induced structure formation was clearly noticed. The effect was larger for GTP; nucleoside monophosphate did not show any effect. When polyphosphate was present, K5 gave a CD spectrum which suggested a large contribution of β-like structure (Fig. 10).

When DOPC were included, the measurements were more difficult due to membrane fusion causing high scattering. In the presence of lipid bilayers, molar ellipticity at 220 nm (as a measure of helicity) increased 21 and 30% for inclusion of ATP or GTP, and polyphosphate, respectively.

Discussion

We reported that the 20-residue peptide E5, having a modified amino-acid sequence of the N-terminal portion of influenza virus hemagglutinin HA-2 subunit, could induce fusion of DOPC LUV [10]. In the case of peptide E5, which had glutamic acid residues arranged according to the principle of amphiphilicity [9], the fusion was induced on lowering the pH of the solution below 6. On the contrary, K5, which is a peptide having the same amino-acid sequence as E5, except for substitution of glutamic acids by lysines, caused similar membrane fusion at pH values higher than 10. Both of the peptides took an α-helical conformation, at least partially, in the presence of lipid bilayers at neutral pH, with enhanced helicity at the pH inducing membrane fusion [9]. The presence of both E5 and K5 induced efficient fusion of LUV at neutral pH, where each peptide was not effective to induce the fusion [10]. On the molecular surfaces, K5 and E5 have an arrangement of positive or negative electric charges complementary to each other when these peptides assumed an α-helical conformation. Association of these two peptides through electrostatic interactions between complementarily arranged charges stabilized an α-helix conformation of the peptides with the concomitant increase of the hydrophobicity of the complex. The fusogenic capacity of these peptides was attributed to an increase of the peptide hydrophobicity and a stabilization of a specific secondary structure [9,10], which were the results of intermolecular neutralization of electric charges on the peptide side chains. A peptide-peptide association of the hydrophilic sides of the α-helices should be also considered as a possible source of an increase of the hydrophobicity.

In the present case, multivalent phosphates substituted the role of E5 in the E5-K5 system and caused K5 to trigger LUV fusion at neutral pH. When K5 took an α-helical conformation, the distances between ϵ-nitrogen atoms of lysine residues are about 4 Å, which matches that of the negatively charged oxygen atoms of oligophosphates (approx. 3.5 Å). Therefore, nucleoside triphosphates or polyphosphate can cross-link the three (possibly four or five when polyphosphate was used) consecutively arranged lysine residues of K5 (Lys^5-Lys^8-Lys^{11}, Lys^8-Lys^{11}-Lys^{15}, etc.) to stabilize the α-helix. The electrostatic nature of the interaction between K5 and oligophosphates was clear, since high salt concentrations cancelled the effect of oligophosphates. A rapid initial aggregation of liposomes induced by K5 and ATP, which was observed by light

scattering and electron microscopy, was also reduced at higher ionic strength (> 0.3 M KCl, Fig. 7). The inhibitory effects of high ionic strength and Mg^{2+} were not due to a lack of association of the peptide to lipids, since the Ficoll floating assay under high ionic strengths or in the presence of $MgCl_2$ showed that the peptide associated with lipid vesicles. The fluorescence of the tryptophyl residue of K5 (emission maximum at 355 nm in the absence of lipids shifted 333 nm in the presence of lipids even with $MgCl_2$ or under high concentrations of KCl) also suggested the interaction of K5 and lipids. The extent of leakage of the LUV contents was nearly unchanged, too, irrespective of the concentration of KCl or the presence of $MgCl_2$. Considering the high ability of Mg^{2+} to form a tight complex with nucleotides (generally with phosphate), the inhibitory effect of $MgCl_2$ at a low concentration is well conceivable.

Interestingly, GTPγS caused K5-mediated fusion but AMP-PNP did not (Fig. 8). Both reagents are nonhydrolyzable nucleoside triphosphate analogs. The result of GTPγS was consistent with the discussion given above, however, that of AMP-PNP was apparently peculiar. Although we cannot give a conclusive explanation for the latter result, it may suggest that the geometry of a bank of phosphate groups (conformation around the P–NH–P bonds) is critical for the interaction.

A requirement of more than a stoichiometric amount of nucleoside tri- or diphosphates to effect LUV fusion (in the case of K5-E5, the optimum molar ratio of K5 and E5 was 1 : 1) and an effective inhibition by the presence of KCl as low as 0.3 M suggested a relatively low association constant of the oligophosphates to K5. For polyphosphate, the less inhibitory effect of added salt means that polyphosphate has a larger association constant to K5 than tri- or diphosphates. Unfortunately, this cannot be discussed in more detail, since we have no knowledge on the distribution of polymerization number of phosphate groups in our polyphosphate sample, however, it is readily conceivable that tetra- or pentaphosphate possibly interacts more strongly with K5, which has five lysine residues.

During the last few decades, conformational transitions of charged polypeptides induced by oppositely charged polyions have been described for many instances. With polyglutamic acid or polylysine, for which most works have been carried out, the importance of charge density of a counter-polyion to induce a conformational transition is well recognized. For example, polylysine forms an ordered secondary structure in the presence of oligoglutamic acid and when the degree of dissociation of the γ-carboxylic groups was around 0.5 [19]. In a system of polylysine and sulfated polyvinylalcohol, α-helix formation of polylysine at neutral pH was dependent on the degree of sulfation [20]. Not only polyions, but also small ions induce ionic polypeptides to adopt ordered secondary structures if the ions were multifunctional. Perchlorate or thiocyanate induced polylysine to take an α-helical conformation at neutral pH [21,22]. Polyarginine α-helix was induced by the presence of perchlorate, sulfate, or phosphate, etc. [23,24]. The formation of a specific and ordered secondary structure could be ascribed to the effect of polyions or multifunctional ions that cross-link the electric charges on the polypeptides. The effectiveness of an ion to induce a characteristic secondary structure of a macromolecule is specific to a macromolecule, the spatial arrangement and the nature of the atoms or atomic groups that carry electric charges on a macromolecule are responsible to the interaction. In the present case, ions such as phosphate, sulfate, citrate, propane-1,2,3-tricarboxylic acid, etc. could not induce K5 to take a stable secondary structure and to trigger membrane fusion. In a preliminary study, we failed to find polycations which act on E5 to induce membrane fusion.

A preliminary mixing of an oligophosphate and K5 prior to the addition of LUV significantly reduced the amount of LUV fusion compared to the case where K5 and an oligophosphate were added successively to an LUV suspension. Because K5 was bound to the liposomes under the conditions, we consider that the result is simply due to the reduction of effective peptide concentration caused by enhanced peptide-peptide association. K5-oligophosphate complexes are extremely hydrophobic, causing increased light scattering of the solution or formation of precipitates.

Yoshimura et al. [25] demonstrated that a model peptide having 51 amino-acid residues in tandem repeats of a Lys-Lys-Leu-Leu sequence, which adopted an amphiphilic α-helix in the presence of ATP, caused fusion of egg-PC LUV at neutral pH. They also suggested that the fusion was triggered when their peptide became hydrophobic by binding of ATP. In the absence of ATP, their peptide neither bound to egg-PC LUV, nor adopted an α-helical structure. Their peptide seemed to form a large hydrophobic aggregates in the presence of ATP and then such aggregated peptide might cross-link the vesicles to cause fusion. On the contrary, under our experimental conditions, K5 bound to egg-PC LUV and adopted an α-helical structure in the absence of ATP (Fig. 10). K5, which bound to vesicles, took an amphiphilic helical structure and they might adopt sterically suitable conformation for binding nucleoside triphosphates.

Düzgüneş et al. [26] have studied the fusion of LUV containing the positively charged lipid *N*-[2,3-(dioleyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium (DOTMA), induced by multivalent anions, such as citrate, phosphate, ethylenediaminetetraacetate, etc. In their case, multivalent anions might induce a complex

formation between positively charged trimethylammonium groups on apposed bilayers of DOTMA/phosphatidylethanolamine vesicles and neutralized the surface charge of vesicles. They suggested that DOTMA-anion-DOTMA complex would be analogous to the dehydrated *trans*-phosphatidylserine-Ca^{2+}-phosphatidylserine complex proposed earlier by Düzgüneş and Papahadjopoulos [27]. The rapid blue shift of tryptophyl fluorescence of K5 within 5 s after an addition of K5 into the LUV suspension and the direct estimation using Ficoll discontinuous gradient suggested the rapid and efficient association of K5 with DOPC vesicles at neutral pH. In the presence of oligophosphates, K5 on the surface of the separate vesicles would be expected to form a hydrophobic complex, K5-oligophosphate-K5 or (K5-oligophosphate)/(K5-oligophosphate) and to induce the fusion in a similar manner to the fusion of DOTMA-containing vesicles. On the other hand, however, the effectiveness of anions in the present case was different from that of the fusion of DOTMA-containing vesicles. EDTA, phosphate, sulfate and citrate, which were effective on the fusion of DOTMA-containing vesicles, were ineffective on K5-mediated fusion.

The fusion of LUV containing various anionic phospholipids induced by the cationic peptides polylysine, polyarginine, polyhistidine, or bee venom melittin has been extensively studied [28–31]. When phosphatidylcholine was included in the vesicles, the fusion was severely inhibited, except for melittin. K5 effectively caused fusion of phosphatidylserine/phosphatidylcholine (1:1 molar ratio) LUV at neutral pH. There are many effectors affecting peptide-induced membrane fusion, such as protons and salts, however, our present results would be a first example for positive regulation of phosphatidylcholine LUV fusion at neutral pH, using such small molecules as oligophosphates.

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