# Specificity of amphiphilic anionic peptides for fusion of phospholipid vesicles

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ABSTRACT We have synthesized five amphiphilic anionic peptides derived from E5 peptide [Murata, M., Takahashi, S., Kagiwada, S., Suzuki, A., Ohnishi, S. 1992. *Biochemistry* 31:1986–1992. E5NN and E5CC are duplications of the N-terminal and the C-terminal halves of E5, respectively, and E5CN is an inversion of the N- and the C-terminal halves. E5P contains a Pro residue in the center of E5 and E8 has 8 Glu residues and 9 Leu residues. We studied fusion of dioleoylphosphatidylcholine (DOPC) large unilamellar vesicles assayed by fluorescent probes. The peptides formed $\alpha$-helical structure with different degrees; E5NN, E5CN, and E8 with high helical content and E5CC and E5P with low helical content. These peptides bound to DOPC vesicles at acidic pH in proportion to the helical content of peptide. The peptides caused leakage of DOPC vesicles which increased with decreasing pH. The leakage was also proportional to the helicity of peptide. Highly helical peptides E5NN, E5CN, and E8 caused hemolysis at acidic pH but not at neutral pH. The fusion activity was also dependent on the helicity of peptides. In fusion induced by an equimolar mixture of E5 analogues and K5 at neutral pH, E8, E5NN, and E5CN were most active but E5CC did not cause fusion. In fusion induced by E5-analogue peptides alone, E5CN was active at acidic pH but not at neutral pH. Other peptides did not cause fusion. Amphiphilic peptides also appear to require other factors to cause fusion.

## INTRODUCTION

We synthesized a twenty-amino acid peptide, called HA-peptide,[1] of the same sequence as that of the N-terminus of HA2 of influenza virus A/PR/8/34 and showed fusion of egg PC sonicated vesicles at acidic pH, in similar manner to fusion of virus (1). We also prepared anionic peptides D4, E5, and E5L and a cationic peptide K5, which are water-soluble analogues of the HA-peptide. K5 had the same amino acid sequence similar to E5, except for 5 Lys residues which replaced 5 Glu residues. All the anionic peptides caused fusion of both egg PC sonicated vesicles and large unilamellar vesicles at acidic pH, and the cationic peptide caused fusion at alkaline pH (2). We also showed that an equimolar mixture of E5 and K5 caused fusion of egg PC LUV at neutral pH (3). These peptides formed $\alpha$-helical structure with amphiphilic nature and were more hydrophobic under the fusion conditions. This is likely due to protonation of the Glu residues at acidic pH, deprotonation of the Lys residues at alkaline pH, or mutual electrostatic interactions between the Glu residues and the Lys residues at neutral pH. These uncharged peptide complexes may interact with closely apposed lipid bilayer membranes to cause fusion.

Lear and DeGrado (4) synthesized a twenty-amino acid peptide from the N-terminus of HA2 of influenza virus B/Lee/40 strain and showed fusion of DOPC sonicated vesicles at neutral and acidic pH values. Wharton et al. (5) also synthesized the 20- and 23-amino acid peptides from influenza virus X-31 strain and indicated fusion of palmitoyl-oleoyl-PC (POPC) vesicles at various pH values. The peptides caused fusion of POPC/cholesterol (1:1) vesicles only at acidic pH. All these studies indicate that the 'fusion peptide' derived from HA was able to cause fusion of phospholipid vesicles but the pH dependence differed between studies. Rafalsky et al. (6) synthesized a 23-amino acid peptide from the N-terminus of "fusion protein" gp41 of human immunodeficiency virus and showed fusion of sonicated phosphatidylglycerol vesicles, but not of PC, at neutral pH. This virus can cause fusion at neutral pH.

In the present study, we design and synthesize five peptides derived from E5 peptide with different characters: E5CN, an inversion of the C-terminal and the N-terminal halves; E5NN, duplication of the N-terminal half only; E5CC, duplication of the C-terminal half only; E5P, with a Pro residue in the 11th position; E8, with 8 Glu residues and 9 Leu residues (Fig. 1). We study fusion of DOPC LUV induced by these amphiphilic anionic peptides assayed by two independent fluorescence methods. We also study fusion induced by an equimolar mixture of E5 analogues and K5 at neutral pH. The results show that E5CN cause fusion at acidic pH. In combination with K5, E8, E5NN, and E5CN cause fusion at neutral pH. Other peptides do not show fusion activities.

## MATERIALS AND METHODS

Peptides were synthesized similar manner to methods previously described (7). The peptide concentration was determined from the absorbance at 280 nm using molar extinction coefficients ($M^{-1}$ $cm^{-1}$) of

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[1] *Abbreviations used in this paper:* HA, hemagglutinin; DOPC, dioleoyl-phosphatidylcholine; LUV, large unilamellar vesicles; NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidyl-ethanolamine; R18, octadecylrhodamine; ANTS, 8-amino-naphthalene-1,2,3-trisulfonic acid; DPX, N,N'-p-xylylene-bis(pyridinium bromide).


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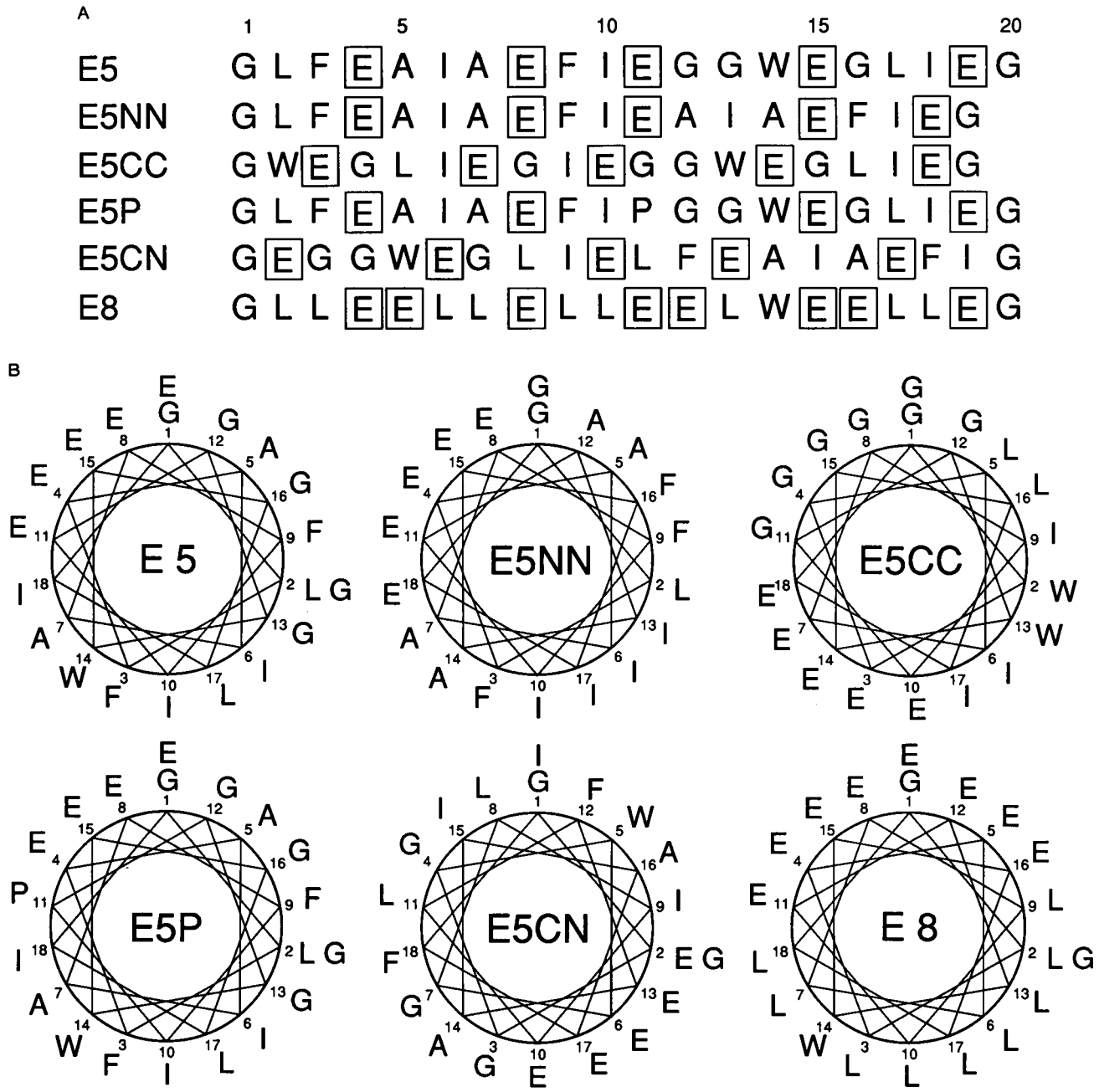


FIGURE 1 (*A*) The primary structure of E5-analogue peptides. (*B*) The helical diagram.

5,000 for E5CN, 11,500 for E5CC, 6,030 for E5P, and 5,500 for E8. E5NN did not contain Trp residues and its concentration was determined by the amino acid analysis. NBD-PE, R18, ANTS, and DPX were purchased from Molecular Probes (Eugene, Oregon). DOPC was obtained from Sigma Chemical Co. (St. Louis, MO). LUV were prepared according to Szoka and Papahadjopoulos (8). Phospholipid concentration was determined by the method of Bartlett (9).

Fusion of DOPC LUV was assayed by lipid mixing (10) and internal content mixing (11) as described (2, 3). Briefly, in the lipid mixing assay, the peptide stock solution was added to a mixture of DOPC/NBD-PE/R18 vesicles (fluorescent probes at 0.75 mol % each) and unlabeled DOPC vesicles at 0.55 mM, each in buffers at various pH values. The lipid to peptide (L/P) ratio was 20–40. The fluorescence intensity of NBD at 530 nm with an excitation at 470 nm was measured at various times after addition of peptide at room temperature (22–23°C) with a Hitachi 850S fluorescence spectrometer. The first point was measured manually at 5 s. The 100% or 0% fusion was taken as the fluorescence intensity of vesicle suspension after addition of Triton X-100 (final 0.3%) or before addition of the peptide, respectively. In the internal content mixing assay, DOPC LUV were prepared in 25 mM ANTS, 40 mM NaCl, 10 mM Tris-HCl or 90 mM DPX, 10 mM Tris-HCl. A mixture of ANTS-containing DOPC vesicles and DPX-containing DOPC vesicles at 0.55 mM each, was added with the peptide and the fluorescence intensity of ANTS at 530 nm with an excitation at 384 nm was measured at various times after addition of peptide. The 0% fusion was the fluorescence intensity of vesicle suspension alone and the 100% fusion was the zero level of the recorder.

Leakage was measured using DOPC LUV prepared in 12.5 mM

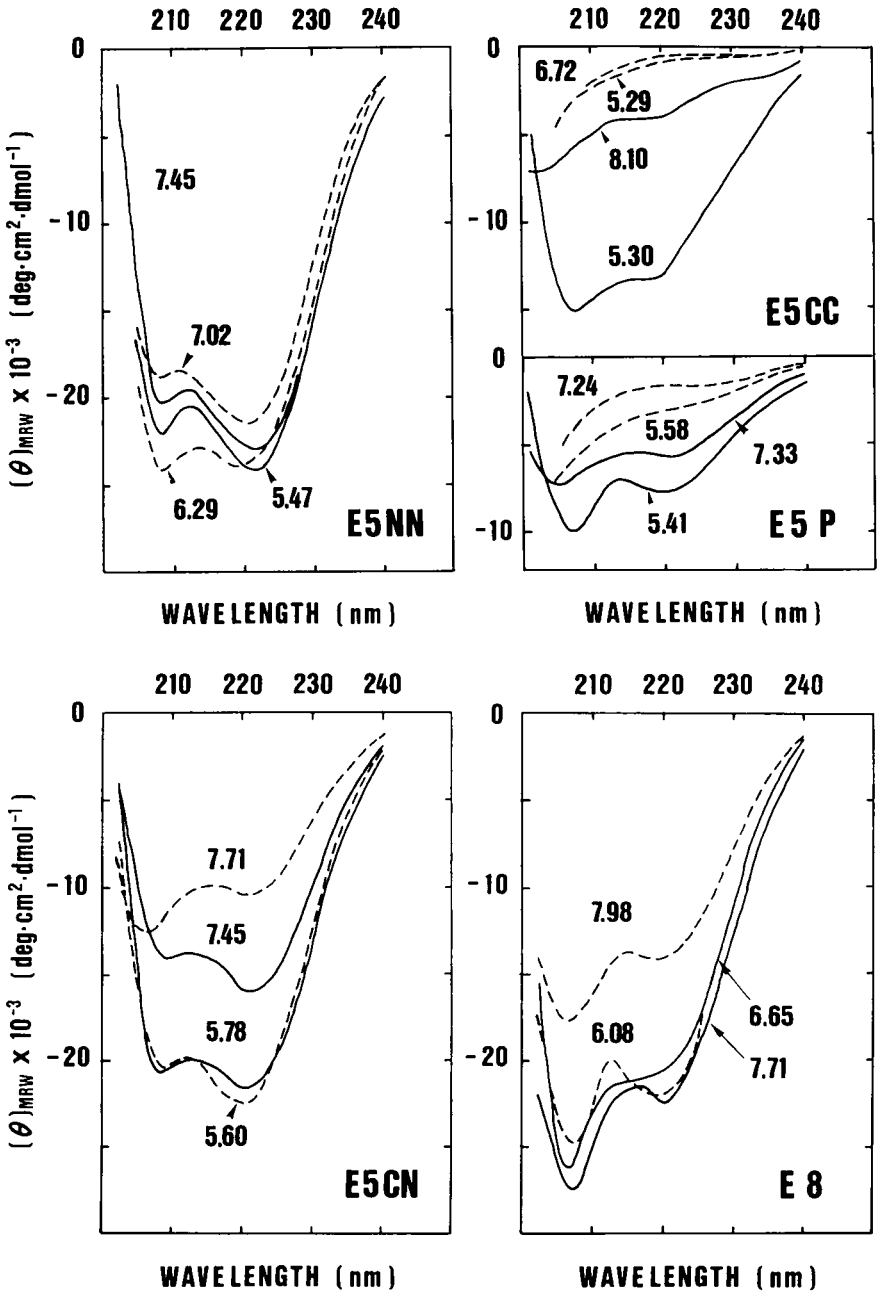


FIGURE 2 The CD spectra of E5-analogue peptides at indicated pH value in the absence (*dotted line*) and presence (*full line*) of DOPC sonicated vesicles. The peptide concentration in the absence of vesicles was 1.55–1.94 mM, except for E8 at 0.25 mM. The peptide concentration was 0.25–0.39 mM and the vesicle concentration was 7.4–8.2 mM, in the presence of vesicles.

ANTS, 45 mM DPX, and 10 mM Tris-HCl. After addition of the peptide solution, the ANTS fluorescence intensity was measured at various times. The 100% leakage was taken as the fluorescence intensity of the vesicle suspension after addition of Triton X-100 (0.3% final). Hemolysis was assayed by addition of peptide (final 6 $\mu$M) to human erythrocytes (5% vol/vol) in phosphate buffer (pH 7.4) at 0°C for 5 min. Isotonic buffers at various pH values were added to the mixture, incubated at 37°C for 30 min, and neutralized by phosphate buffer. The optical density at 520 nm was measured.

Binding of peptides to DOPC LUV was assayed by flotation centrifugation with Ficoll (Nacalai Tesque, Kyoto, Japan) according to Shen et al. (12). After incubation of peptide and DOPC LUV containing 1 mol% of NBD-PE for 10 min at room temperature, Ficoll was added to the mixture and then centrifuged as described (2). The peptide concentration was determined by the fluorescamine assay and the vesicle concentration by the NBD fluorescence intensity.

The fluorescence spectrum of the Trp residue of the peptides (5.6–28 $\mu$M) was measured in the absence and presence of DOPC sonicated vesicles at 1.4 mM. The 90° light scattering of vesicle suspension was measured at 400 nm. The CD spectra of peptides in the absence and presence of DOPC sonicated vesicles were measured with a JASCO J-20 spectropolarimeter (JASCO International Co. Ltd., Hachioji City, Japan). The ellipticity was expressed as the mean residue weight basis.

## RESULTS

*Design principle for E5-analogue peptides.* The fusion-active E5 peptide has an amino acid sequence in

TABLE 1 **The ellipticity value $\vartheta \times 10^{-3}$ at a minimum near 222 nm in CD spectrum of E5-analogue peptides**

| pH | Vesicles | E5 | E5CN | E5NN | E5CC | E5P | E8 |
|---|---|---|---|---|---|---|---|
| neutral | absence | small | −11 | −21 | small | small | −19 |
| | presence | −13 | −16 | −23 | −4 | −6 | −21 |
| acidic | absence | −22 | −22 | −24 | small | small | −22 |
| | presence | −20 | −22 | −24 | −13 | −8 | −22 |

which the N- and C-terminal halves apparently have different characteristics (Fig. 1). The sequence 1–10 has a greater propensity for $\alpha$-helix than that for $\beta$-structure based on a statistical evaluation (13, 14); in contrast, the reverse is true for sequence 11–20. In an attempt to localize an actively contributing domain in E5, we divided the amino acid sequence into the N- and C-terminal halves and prepared several peptides with different combinations. In the peptides E5NN and E5CC, the residue sequence of the N- and C-terminal halves were duplicated, respectively, to make the length of these peptides comparable to that of E5 under a restriction of amphiphilicity. E5NN was anticipated to be highly helical and E5CC to be a less ordered structure. A Pro residue was inserted into E5P to interrupt the secondary structure in E5. Interchange of the N- and C-terminal halves of E5 gave E5CN, in which a Glu residue occupied position 2, instead of 4 as in E5. Since a negative charge located near the N-terminus stabilizes the helix structure, E5CN was expected to be more $\alpha$-helical than E5. E8 contained more Leu and Glu residues.

*CD spectra of E5-analogue peptides.* CD spectra of peptides at various pH values in the absence and presence of DOPC sonicated vesicles are shown in Fig. 2. These peptides formed $\alpha$-helical structure which was dependent on the peptide. The helical content was increased with decreasing pH values, with the peptide concentrations, and by addition of phospholipid vesicles, as shown for E5 (2, 15).

E5NN, E5CN, and E8 formed high degrees of $\alpha$-helical content (Fig. 2), as expected. The ellipticity value $\vartheta \times 10^{-3}$ at 222 nm was −24 for E5NN and −22 for E5CN and E8 at acidic pH as shown in Table 1. The ellipticity value was smaller at neutral pH (Table 1). E5CC and E5P were nearly random coil in the absence of vesicles. However, in the presence of vesicles, they formed $\alpha$-helical structure to some extent (Fig. 2). The ellipticity value $\vartheta \times 10^{-3}$ was −8 for E5P and −13 for E5CC, about 33% for E5P and 54% for E5CC as compared with that for E5NN (Table 1). The ellipticity value at acidic pH in the presence of vesicles is tabulated symbolically in Table 3.

*Binding of peptides to DOPC LUV.* Binding was measured at various pH values by flotation on the discontinuous Ficoll gradients. E5-analogue peptides did not bind to DOPC LUV at neutral pH, in contrast to E5. At acidic pH, E5NN bound vesicles strongly to nearly the same extent as E5 (2). E5CN, E5P, and E8 also bound but weaker than E5NN. The binding was approximately 50% for E5CN and 20% for E5P and E8 of that for E5NN, as estimated by the fluorescamine assay (see Table 3).

The Trp fluorescence spectrum of E5-analogue peptides shifted to a lower wavelength by addition of DOPC vesicles. Peptides alone showed the fluorescence maximum at 350–356 nm at neutral pH as shown in Table 2. At acidic pH, most of them showed similar wavelengths but E5CN was shifted to a lower wavelength with the maximum at 337.3 nm (from 350.0 nm at neutral pH). $\Delta\nu_1$ in Table 2 shows the shift by acidification for peptide alone. The fluorescence maximum of E5 analogues in the presence of vesicles was blue-shifted to 330–345 nm at acidic pH. If we express the blue shift by $\Delta\nu_3$, which is the difference of the maximum wavelength of peptide alone at pH 7.4 minus that of peptide/DOPC at pH 5.0, it was 16.3 nm for E5CN, 8.9 nm for E5CC, 13.8 nm for E5P, and 25.3 nm for E8 as shown in Table 2. If we use the blue shift by $\Delta\nu_2$ for the difference of the maximum wavelength of peptide alone at pH 5.0 minus that of peptide/DOPC at pH 5.0 (Table 2), the small value 3.6 nm for E5CN arises from the acid-induced blue shift of E5CN alone as described above. $\Delta\nu_3$ was symbolically tabulated in Table 3.

*Fusion induced by E5-analogue peptides.* Fusion of DOPC LUV induced by peptides was measured by two independent fluorescence methods. In the lipid mixing assay, a mixture of DOPC/NBD-PE/R18 vesicles and unlabeled DOPC vesicles was incubated with peptide. The NBD fluorescence intensity should increase upon fusion because of dilution of NBD-PE and R18 with phospholipids of unlabeled vesicles. In the internal content mixing assay, a mixture of ANTS-containing vesicles and DPX-containing vesicles was incubated with peptide, the ANTS fluorescence intensity should decrease because of the complex formation of ANTS and DPX in fused vesicles.

TABLE 2 **The maximum wavelength for fluorescence spectrum of Trp residue of E5-analogue peptides in the absence and presence of DOPC sonicated vesicles**

| | Absence of vesicles | | Presence of vesicles | | | | |
|---|---|---|---|---|---|---|---|
| | pH 7.4 | pH 5.0 | pH 7.4 | pH 5.0 | $\Delta\nu_1$ | $\Delta\nu_2$ | $\Delta\nu_3$ |
| E5CC | 354.2 | 351.3 | 352.9 | 345.3 | 2.9 | 6.0 | 8.9 |
| E5P | 353.1 | 352.3 | 346.6 | 339.3 | 0.8 | 13.0 | 13.8 |
| E5CN | 350.0 | 337.3 | 344.4 | 333.7 | 12.7 | 3.6 | 16.3 |
| E8 | 356.1 | 350.5 | 351.1 | 330.8 | 5.6 | 19.7 | 25.3 |

$\Delta\nu_1$ = (peptide alone pH 7)–(peptide alone pH5); $\Delta\nu_2$ = (peptide alone pH 5)–(peptide/DOPC pH 5); $\Delta\nu_3$ = (peptide alone pH 7)–(peptide/DOPC pH 5). The L/P ratio = 50.

TABLE 3 Function of E5-analogue peptides on DOPC LUV or red blood cells at acidic pH

| | α-Helix | Binding | Blue shift of Trp fluorescence | Fusion induced by | | Leakage | Hemolysis |
|---|---|---|---|---|---|---|---|
| | | | | Peptides | Peptides and K5 at neutral pH | | |
| E5 | +++ | +++ | ++ | +++ | ++ | ++++ | ++ |
| E5CN | +++ | ++ | ++ | ++ | ++ | ++++ | +++ |
| E5NN | +++ | +++ | nd | − | ++ | ++ | +++ |
| E5CC | + | − | + | − | − | − | − |
| E5P | + | + | ++ | ± | ± | ± | − |
| E8 | +++ | + | +++ | − | +++ | +++ | +++ |

Of the E5-analogue peptides, E5CN caused fusion at acidic pH but not at neutral pH. In lipid mixing assay (Fig. 3 *Ba*), the fluorescence intensity initially increased rapidly and then became more gradual at acidic pH. The fusion was 11% of the maximum in the initial phase. In the internal content mixing assay (Fig. 3 *Bb*), the ANTS fluorescence intensity decreased rapidly and then became more gradual at acidic pH. The fusion was estimated as 12% of the maximum in the initial phase. Nearly the same results were obtained from the two independent fluorescence assays, confirming the fusion event. The experiments for E5 were also plotted in Fig. 3 *A*. The fusion induced by E5 was 15% of the maximum by the lipid mixing assay and 9% of the maximum by the internal content mixing assay in the initial phase at acidic pH. E8 showed a slight and slow increase in the NBD fluorescence intensity. The fusion was 5% of the maximum after 2 min. However, E5NN, E5CC, and E5P did not cause fusion at neutral and acidic pH values. The pH dependence of fusion extent induced by E5 analogues after 5 min is given in Fig. 4 and summarized in Table 3.

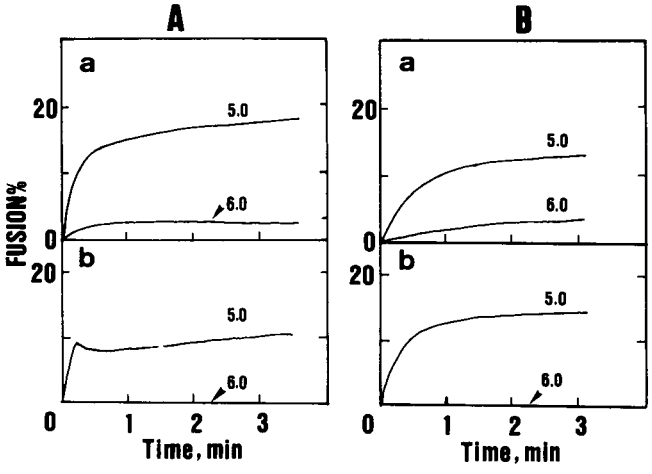


FIGURE 3 The time course of fusion of DOPC LUV induced by E5 (*A*) or E5CN (*B*) at indicated pH value assayed by lipid mixing (*a*) and internal content mixing (*b*). The vesicle concentration was 1.1 mM and the L/P ratio was 41.

The 90° light scattering of a suspension of E5-analogue peptides and DOPC LUV was small at neutral pH. When the pH was lowered, the scattering from DOPC LUV and E5CN, but not other E5 analogues, increased significantly, indicating vesicle aggregation and fusion.

*Fusion induced by an equimolar mixture of E5-analogue peptides and K5.* We have shown previously that an equimolar mixture of E5 and K5 caused fusion of egg PC LUV at neutral pH, by electrostatic interactions between the two peptides (3). Therefore, the fusion ability of an equimolar mixture of E5 analogues and K5 at

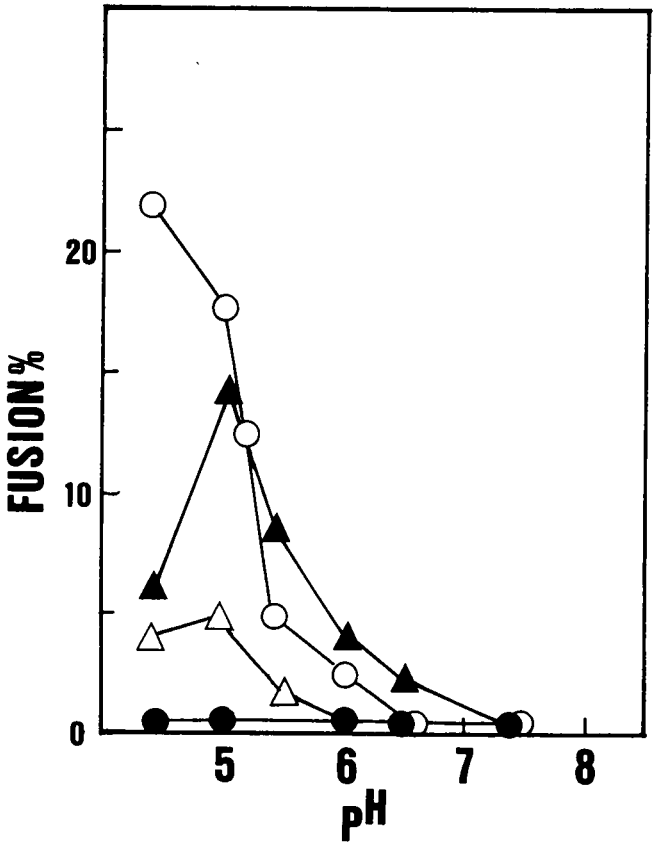


FIGURE 4 The pH dependence of fusion extent of DOPC LUV by E5-analogue peptides after 5 min assayed by lipid mixing; (○) E5, (▲) E5CN, (△) E8 and (●) E5NN, E5CC, and E5P.

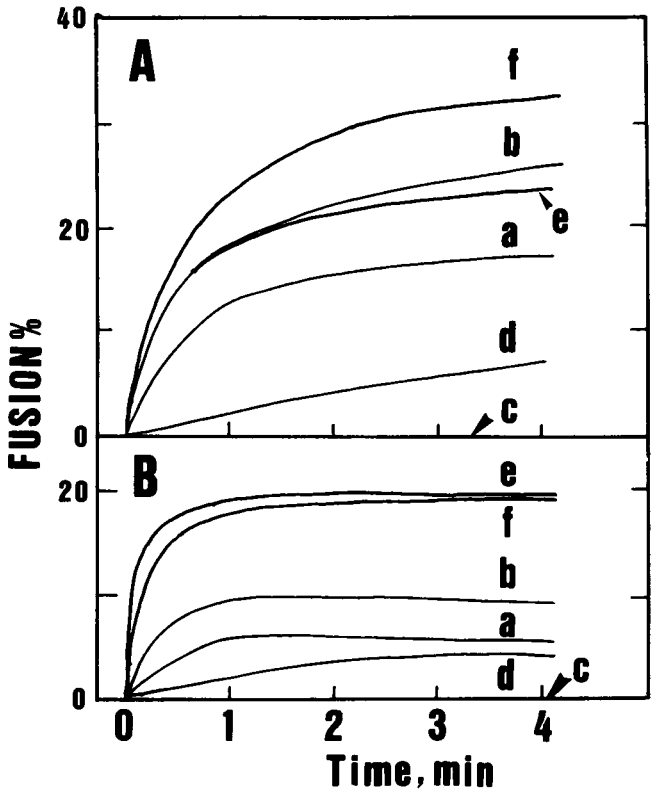


FIGURE 5 The time course of fusion of DOPC LUV induced by an equimolar mixture of K5 and E5-analogue peptides at pH 7.4 assayed by lipid mixing (*A*) and internal content mixing (*B*); (*a*) E5, (*b*) E5NN, (*c*) E5CC, (*d*) E5P, (*e*) E5CN, and (*f*) E8. E5-analogue peptides and K5 at 13 $\mu$M each were added to DOPC LUV (1.1 mM (*A*) and 0.82 mM (*B*)) within 2 sec and the fluorescence intensity was measured.

neutral pH was investigated. In the lipid mixing assay (Fig. 5 *A*), E8 and K5 caused fusion most extensively at 33% of the maximum after 4 min. The fusion efficiency was a little larger than that of E5 (18%). E5NN and E5CN, in combination with K5, also caused fusion extensively at 23% of the maximum. E5P and K5 showed a slow and small fusion of 5% of the maximum after 4 min. E5CC and K5 did not cause fusion. The fusion efficiency was summarized in Table 3. In internal content mixing assay (Fig. 5 *B*), the fusion was smaller than that assayed by lipid mixing.

The E5-analogue peptides, except for E5CC, were able to cause fusion when combined with K5 at neutral pH. This is in contrast to fusion by E5CN at acidic pH when the peptide alone was added to vesicles.

The 90° light scattering of DOPC LUV was increased largely when an equimolar mixture of K5 and E5CN, E5NN, E5P, or E8 was added to vesicle suspension at neutral pH. However, E5CC and K5 increased the light scattering only slightly. The increase in light scattering paralleled with the fusion activity.

*Leakage and hemolysis induced by E5-analogue peptides.* Leakage of ANTS/DPX from DOPC LUV induced by E5-analogue peptides was measured. The leakage was dependent on the peptide, greater at acidic pH and less at neutral pH. The most extensive leakage was induced by E5CN. The ANTS fluorescence intensity increased to about 70% of the maximum immediately after addition of peptide at acidic pH (Fig. 6). The leakage efficiency was similar to that of E5. The leakage at neutral pH was much less and about 30% of the maximum after 3 min. The fusogenic peptides therefore cause leakage. Both rates were very large initially after addition of peptide to vesicles. The fusion was probably faster than the leakage. It was shown that similar extents of fusion were obtained by lipid mixing as well as content mixing. Some data such as E5CN (Fig. 6) showed larger rates of leakage than that of fusion (Fig. 3 *Bb*). This may arise from the first point of measurement being done manually at 5 s (see also reference 3).

E8 also caused an extensive leakage of 60% of the maximum after 3 min at acidic pH. The leakage rate was slower than that of E5CN as shown in Fig. 6. E5NN caused a leakage of 35% of the maximum at acidic pH. However, the leakage induced by E5P was very small and none was induced by E5CC (Fig. 6). The pH dependence of leakage after 5 min, induced by E5 analogues is plotted in Fig. 7 and tabulated in Table 3. The leakage experiments were carried out at a L/P ratio of 41. When the L/P ratio was changed from 27 to 82, the leakage was larger with smaller ratios.

Leakage induced by a combination of K5 and E5- analogue peptides at neutral pH was also measured. The leakage amount was dependent on peptide. The leakage was most extensive for E5NN (70% of the maximum after 3 min), large for E5P (50% of the maximum), and very small for E5CC. The leakage efficiency for E5 and K5 was about 80% of the maximum after 4 min.

Hemolysis induced by E5-analogue peptides at various pH values was measured. E8, E5CN, and E5NN induced extensive hemolysis at acidic pH but not at neutral pH (Fig. 8 *A*). E5 also caused hemolysis at acidic pH. However, E5CC and E5P did not hemolyze at both pH values. The hemolysis activity is tabulated in Table 3.

When smaller concentrations of peptide were added to red blood cell suspension, the hemolytic peptides showed a different dose-dependence (Fig. 8 *B*). E8 and E5CN showed the maximum hemolysis at 1 $\mu$M and 2.8 $\mu$M, respectively, but E5NN gradually increased, inducing 50% hemolysis at 4.2 $\mu$M.

## DISCUSSION

The E5-analogue peptides are amphiphilic anionic peptides with different degrees of $\alpha$-helical content and show different effects on DOPC vesicles or red blood cells as summarized in Table 3. The peptides with a high degree of helical content appear to bind to DOPC vesicles and cause leakage, hemolysis, and fusion at acidic pH. The

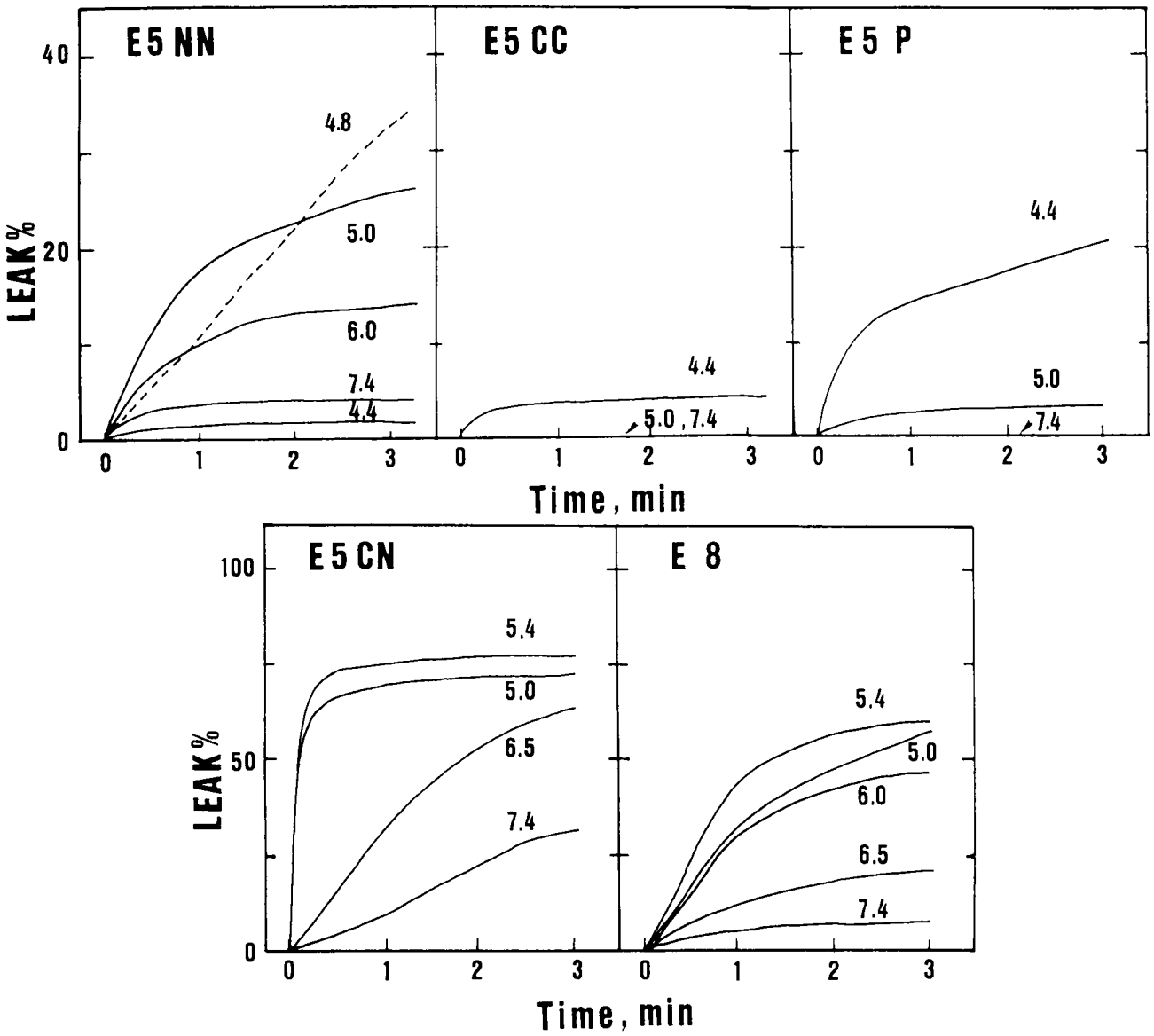


FIGURE 6 The time course of leakage of DOPC LUV containing ANTS/DPX induced by E5-analogue peptides at indicated pH value. The vesicle concentration was 0.82 mM and the L/P ratio was 41.

helical peptides in combination with cationic peptide K5 cause fusion at neutral pH.

The $\alpha$-helical content is high for E5NN, E5CN, and E8, in addition to E5. However, the content for E5CC and E5P is low. Such dependency of helicity is quite easily understood from the discussion for design principle in the Results section. The N-terminal half of E5 had a higher ability to form helicity than the C-terminal half. The small helical content for E5P was due to the presence of Pro residue in the center. Many Leu and Glu residues in E8 caused helical content to increase. The E5 analogues, in addition to D4, E5, and E5L, had higher degrees of helicity in lower pH values, at higher peptide concentrations, and by addition of vesicles. Therefore, the helical structure may be induced by protonation of the Glu residues and by interactions with the peptides and with the phospholipid membranes.

Binding of E5-analogue peptides to DOPC LUV was measured using the discontinuous Ficoll gradients, as carried out for E5, E5L, D4, and K5 (2). The latter peptides bound strongly at neutral and acidic pH values. Of the E5-analogues, E5NN bound strongly to DOPC as E5 at acidic pH. E5CN, E8, and E5P bound to lesser extents and E5CC did not bind at acidic pH. These peptides did not bind at neutral pH. Therefore, the E5-analogue peptides bound to vesicles weaker than E5. There was a blue shift of the Trp fluorescence spectrum by addition of vesicles. Even E5CC showed the blue shift, suggesting binding to vesicles. The amino acid arrangement in helical structure may be important for interaction with phos-

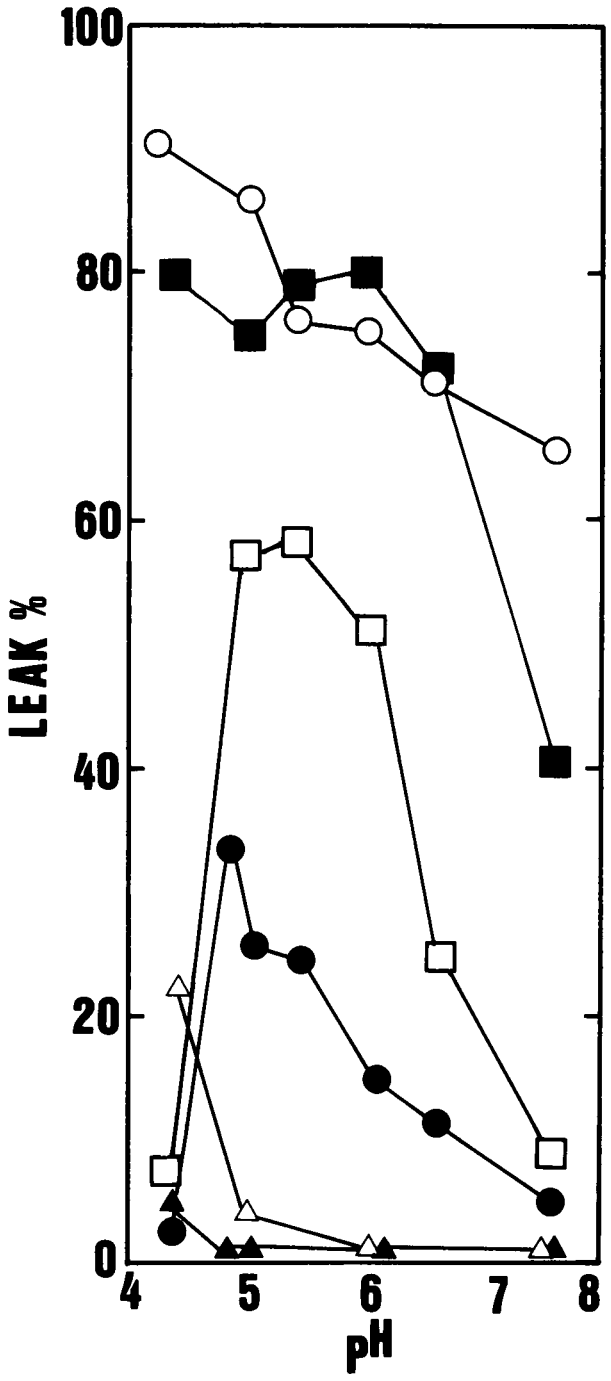


FIGURE 7 The pH dependence of leakage amount of DOPC LUV containing ANTS/DPX induced by E5-analogue peptides measured after 5 min; (○) E5, (●) E5NN, (▲) E5CC, (△) E5P, (■) E5CN, and (□) E8.

pholipid membranes because of stronger binding by more helical peptides.

Leakage and hemolysis activities are dependent on the α-helicity of E5-analogue peptides. E5CN, E8, and E5NN with high helical content caused extensive leakage and hemolysis at acidic pH. However, E5CC did not cause leakage and hemolysis. Parente et al. (16) showed such correlation for an amphipathic 30-amino acid peptide GALA, which caused leakage of egg PC LUV at pH 5.0, where GALA formed α-helical structure. Greater leakage of vesicles was induced by much smaller concentrations of GALA at the L/P ratio of 500–3000 than by E5 analogues at the L/P ratio of 27–82. These authors suggested that 8–12 GALA molecules formed pores as a transbilayer channels and caused leakage (17). There is a possibility for pore formation for several E5 analogues and D4, E5, and E5L. Wharton et al. (5) showed hemolysis induced by the 20-amino acid peptide from influenza virus X-31 strain. The peptide induced hemolysis much greater at acidic pH and lesser at neutral pH. The peptide alone did not assume α-helical structure at various pH values. In the presence of POPC vesicles, it formed helical structure at acidic and neutral pH values. In the presence of POPC/cholesterol vesicles, the peptide formed helical structure only at acidic pH. A 17-amino acid peptide derived from the N-terminus of influenza virus X-31 strain did not cause fusion of sonicated PC vesicles but caused leakage at acidic and neutral pH values (18). This peptide did not form α-helical structure. The 25-amino acid peptide from the N-terminus of vesicular stomatitis virus G protein caused hemolysis at acidic pH. The 6-amino acid peptide from the N-terminus also induced hemolysis at neutral and acidic pH values (19).

There is a correlation between the α-helicity of E5-analogue peptides and the fusion activity. This is especially evident for fusion induced by a combination of K5 and E5 analogues at neutral pH. E8, E5NN, and E5CN, in addition to K5, caused fusion extensively. However, E5P fused slightly and E5CC did not. The fusion mechanism by an equimolar mixture of E5 and K5 was discussed

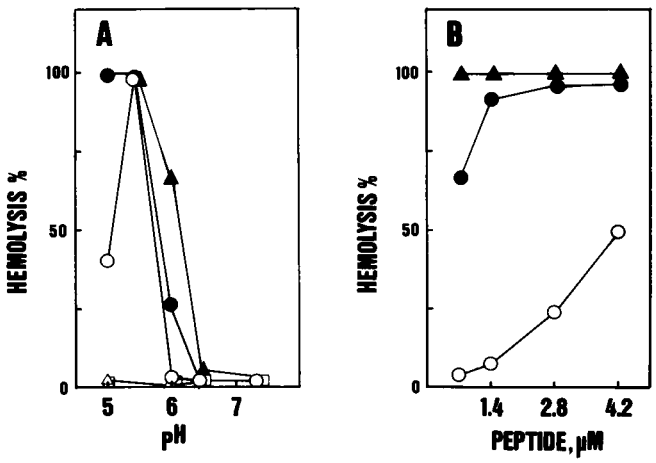


FIGURE 8 (*A*) The pH dependence of hemolysis extent induced by E5-analogue peptides 30 min at 37°C; (○) E5NN, (●) E5CN, (▲) E8, (△) E5CC, and (□) E5P. The peptide concentration was final 5 μM. (*B*) Dose-dependence of hemolysis induced by E5NN (○), E5CN (●), and E8 (▲). The pH value was 5.0 for E8 and E5CN, and 5.4 for E5NN.

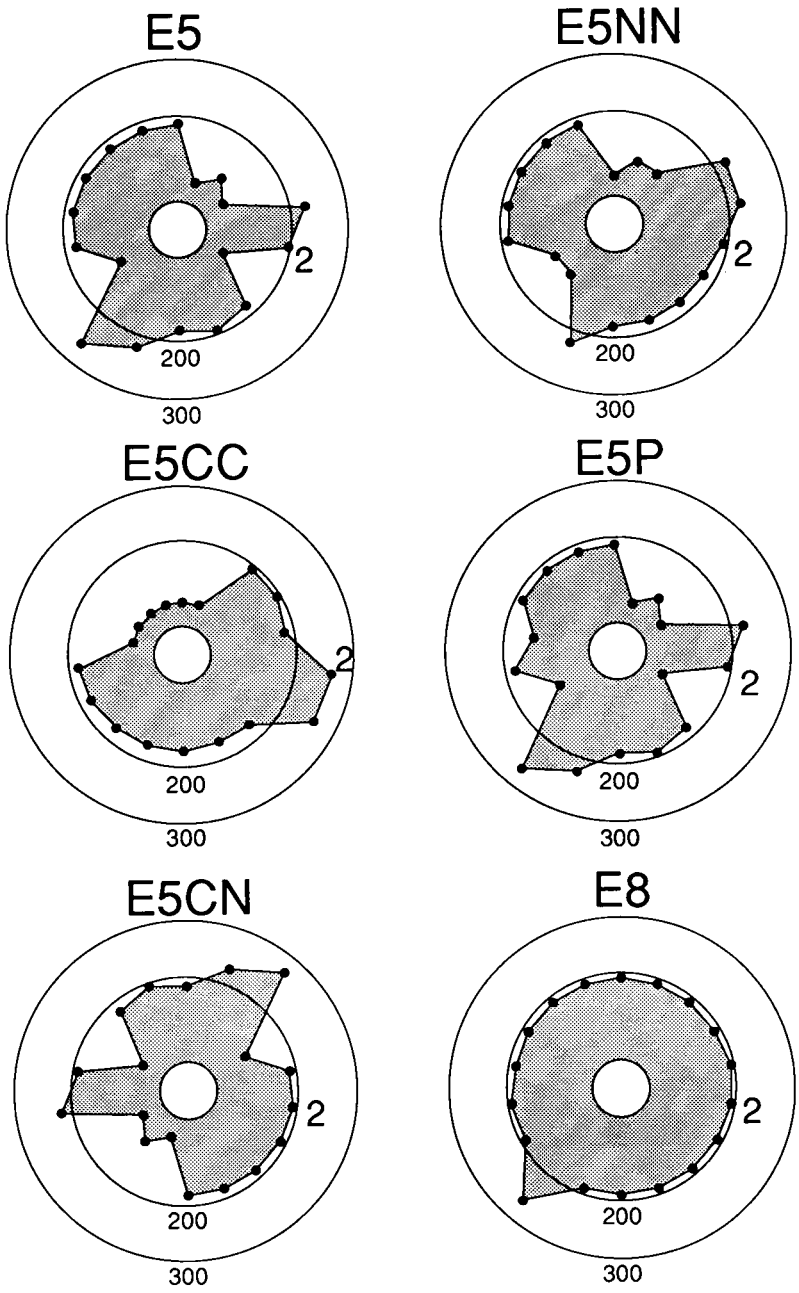


FIGURE 9 Plots of the solvent-accessible surface area for residue side chains of E5-analogue peptides. The values of the area, which were calculated for side chains in an extended conformation (21), are plotted at the residue positions 2–19 in a helical wheel representation. The scale ($A^2$) is in the radial direction.

previously (3). The two peptides formed a complex with each other by electrostatic interaction between the negative charges of Glu residues and the positive charges of Lys residues at neutral pH. The complex had zero net charge, formed $\alpha$-helical structure, and would interact directly with lipid bilayer membranes. Of the E5 analogues, highly helical E8, E5NN, and E5CN can form similar complexes with K5, although some amino acid residues are different from K5. E5P may form partial helical structure by interacting with $\alpha$-helical K5, thus causing smaller fusion. E5CC may not form such complexes by interacting with K5.

The fusion activity of E5-analogue peptides alone was also dependent on the $\alpha$-helicity. However, E5CN was active at acidic pH. However, helical E8 caused only slight fusion and E5NN did not cause any fusion. The correlation of fusion activity and $\alpha$-helical structure was shown by various authors, as for example, (4). Harter et al. (20) indicated that HA inserted its fusion peptide adopting an amphiphilic helical structure in the outer

leaflet of lipid bilayers at acidic conditions. However, the present results indicate that amphiphilic helical peptides also require other factors to cause fusion. One characteristic of fusion-active peptides appears to be their uneven distribution of the side chain bulkiness over the peptides. For example, we considered the solvent-accessible surface area for the side chains (21) as a measure of the side chain bulkiness. These are the values of surface area of side chains which are in the extended conformation and also finally well-correlated with the hydrophobicity of resides (22). As shown in Fig. 9, when a peptide assumed an $\alpha$-helical conformation and was observed from the helical axis, an envelope connecting the surface area values assigned to the residue positions is rough in the case of fusion-active peptides; in contrast, it is rather smooth for fusion-inactive peptides. When such a "rough" peptide, in which the bulky side chains are distributed unevenly, was placed in a lipid bilayer membrane and in dynamic motion, much more perturbations on lipid structure could be expected than that for a "smooth" peptide. Similar analysis for bulkiness is being studied for other fusion proteins and mutants.

Brasseur et al. (23) recently studied theoretically the conformation and mode of insertion of the "fusion peptide" from various virus strains to a lipid monolayer. The N-terminal peptide was inserted as an $\alpha$-helical structure into dipalmitoyl-PC monolayers and oriented obliquely with respect to the lipid/water interface. The unusual orientation may be a prerequisite to membrane destabilization and fusogenic activity.

E5CN caused fusion at acidic pH. The fusion ability at acidic pH but not at neutral pH has also been observed for amphiphilic anionic peptides of HA-peptide, D4, E5, and E5L interacted with sonicated and large unilamellar PC vesicles (1, 2). This may be reasonable since insertion of the carboxyl groups ($-$ COOH) of Glu and Asp residues into phospholipid bilayer hydrocarbon layers is more stable due to a decrease in Gibbs free energy (about 40 kJ/mol) than that for insertion of anionic carboxyl groups ($-$ $COO^-$) of acidic residues as suggested by Ohnishi (24). Our suggestion was that the amphiphilic peptides were more helical and hydrophobic by protonation of Glu or Asp residues at acidic pH, by deprotonation of Lys residues at alkaline pH, or by mutual interactions of two peptides at neutral pH. These peptides were aggregated vesicles, entered closely apposing lipid bilayer membranes more easily and caused fusion (2, 3). A possibility is to add charged molecules into membranes in order to change the pH dependence for fusion induced by amphiphilic peptides. When we used phosphatidylserine/DOPC membranes (1:1) LUV, K5 caused fusion not only at alkaline pH (3) but also at neutral and acidic pH values (Murata, M., S. Takahashi, Y. Shirai, S. Kagiwada, R. Hishida, and S. Ohnishi, unpublished observation). Lear and DeGrado (4) showed fusion of DOPC sonicated vesicles at neutral and acidic pH values. Wharton et al. (5) showed fusion of POPC vesicles at both pH values, larger at acidic pH and smaller at neutral pH. When cholesterol was added to POPC membranes at 1:1 molar ratio, the fusion occurred only at acidic pH. Against this background, we pose the question: What is the molecular mechanism to change pH dependence for fusion by adding neutral cholesterol to neutral DOPC membrane?

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## REFERENCES

1. Murata, M., Y. Sugahara, S. Takahashi, and S. Ohnishi. 1987. pH-dependent membrane fusion activity of a synthetic twenty amino acid peptide with the same sequence as that of the hydrophobic segment in influenza virus hemagglutinin. *J. Biochem.* 102:957–962.
2. Murata, M., S. Takahashi, S. Kagiwada, A. Suzuki, and S. Ohnishi. 1992. pH-dependent membrane fusion and vesiculation of phospholipid large unilamellar vesicles induced by amphiphilic anionic and cationic peptides. *Biochemistry* 31:1986–1992.
3. Murata, M., S. Kagiwada, S. Takahashi, and S. Ohnishi. 1991. Membrane fusion induced by mutual interaction of the two charge-reversed amphiphilic peptides at neutral pH. *J. Biol. Chem.* 266:14353–14358.
4. Lear, J. D., and W. F. DeGrado. 1987. Membrane binding and conformational properties of peptides representing the NH2 terminus of influenza HA-2. *J. Biol. Chem.* 262:6500–6505.
5. Wharton, S. A., S. R. Martin, R. W. H. Ruigrok, J. J. Skehel, and D. C. Wiley. 1988. Membrane fusion by peptide analogues of influenza virus haemagglutinin. *J. Gen. Virol.* 69:1847–1857.
6. Rafalski, M., J. D. Lear, and W. F. DeGrado. 1990. Phospholipid interactions of synthetic peptides representing the N-terminus of HIV gp41. *Biochemistry.* 29:7917–7922.
7. Takahashi, S. 1990. Conformation of membrane fusion-active 20-residue peptides with or without lipid bilayers. Implication of $\alpha$-helix formation for membrane fusion. *Biochemistry.* 29:6257–6264.
8. Szoka, F., Jr., and D. Papahadjopoulos. 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. USA.* 75:4194–4198.
9. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 243:466–468.
10. Struck, D., D. Hoekstra, and R. E. Pagano. 1981. Use of resonance energy transfer to monitor membrane fusion. *Biochemistry.* 20:4093–4099.
11. Ellens, H., J. Bentz, and F. C. Szoka. 1984. pH-induced destabilization of phosphatidylethanolamine-containing liposomes: Role of bilayer contact. *Biochemistry.* 23:1532–1538.
12. Shen, D.-F., A. Huang, and L. Huang. 1982. An improved method for covalent attachment of antibody to liposomes. *Biochim. Biophys. Acta.* 689:31–37.
13. Chou, P. Y., and G. D. Fasman. 1974*a*. Conformational parame-

ters for amino acids in helical, β-sheet, and random coil regions calculated from proteins. *Biochemistry.* 13:211–222.

14. Chou, P. Y., and G. D. Fasman. 1974*b*. Prediction of protein conformation. *Biochemistry.* 13:222–245.
15. Murata, M., S. Takahashi, T. Kanaseki, and S. Ohnishi. 1990. Membrane fusion and lysis by the amphiphilic peptides by charge neutralization: A model study of virus membrane fusion mechanism. *In* Biophysics of the Cell Surface. N. Glaser and D. Gingell, editors. Springer-Verlag, Berlin. 221–236.
16. Parente, R. A., S. Nir, and F. Szoka, Jr. 1988. pH-dependent fusion of phosphatidylcholine small vesicles. Induction by a synthetic amphipathic peptide. *J. Biol. Chem.* 263:4724–4730.
17. Parente, R. A., S. Nir, and F. C. Szoka, Jr. 1990. Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. *Biochemistry.* 29:8720–8728.
18. Duzgunes, N., and F. Gambale. 1988. Membrane action of synthetic N-terminal peptides of influenza virus hemagglutinin and its mutants. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 227:110–114.
19. Schlegel, R., and M. Wade. 1985. Biologically active peptides of the vesicular stomatitis virus glycoprotein. *J. Virol.* 53:319–323.
20. Harter, C., P. James, T. Bachi, G. Semenza, and J. Brunner. 1989. Hydrophobic binding of the ectodomation of influenza hemagglutinin to membranes occurs through the "fusion peptide." *J. Biol. Chem.* 264:6459–6464.
21. Rose, G. D., A. R. Geselowitz, G. J. Lesser, R. H. Lee, and M. H. Zehfus. 1985. Hydrophobicity of amino acid residues in globular proteins. *Science (Wash. DC).* 229:834–838.
22. Cornette, J. L., K. B. Cease, H. Margalit, J. L. Spouge, J. A. Berzofsky, and C. DeLisi. 1987. Hydrophobicity scales and computational techniques for detecting amphiphilic structures in proteins. *J. Mol. Biol.* 195:659–685.
23. Brasseur, R., M. Vandenbranden, B. Cornet, A. Burny, and J.-M. Ruysschaert. 1990. Orientation into the lipid bilayer of an asymmetric amphipathic helical peptide located at the N-terminus of viral fusion proteins. *Biochim. Biophys. Acta.* 1029:267–273.
24. Ohnishi, S. 1988. Fusion of viral envelopes with cellular membranes. *Curr. Top. Membr. Transp.* 32:257–296.