Interaction of Fusogenic Synthetic Peptide with Phospholipid Bilayers: Orientation of the Peptide α-Helix and Binding Isotherm†

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ABSTRACT: We studied the binding characteristics of a synthetic 20-residue peptide to supported single planar bilayers of phosphatidylcholine, and the orientation of the peptide by Fourier-transform infrared spectroscopy with an attenuated total reflection method. This peptide, designed to resemble a putative fusion peptide of influenza virus hemagglutinin, assumes an amphiphilic α-helix and induces fusion of liposomes in an acidic solution (pH ∼5). At neutral pH, the peptides were bound to lipid bilayers in the manner of a Langmuir's adsorption isotherm, and their orientation was nearly random or oblique. On the other hand, at acidic pH, the peptides were bound, making their helix axis parallel to the membrane surface, and the binding was cooperative. This cooperativity suggested dimerization of the peptides. These characteristics are expected to be important for the synthetic fusogenic peptide or the fusion peptide in hemagglutinin to induce membrane fusion.

Membrane fusion is known to be an important step in various inter- and intracellular transport processes of material in the life of cells. For example, viral infection occurs as a result of the transport of a viral genome into a host cell, caused by membrane fusion between virus and cells (White et al., 1983; Stegmann et al., 1989; White, 1992; Zimmerberg et al., 1993). In the case of influenza, which is one of the most investigated examples, fusion is induced by a viral envelope glycoprotein, hemagglutinin, which is inactive at neutral pH. Activation of the protein of an endocytosed virus occurs on lowering the pH of the endosome (Maeda et al., 1980, 1981; Doms et al., 1985, 1986). Many studies have revealed that a large amount of steric structure change in the protein is associated with a pH change or with an expression of the activity (Skehel et al., 1982; Ruigrok et al., 1986; White et al., 1987; Wharton et al., 1988; Stegmann et al., 1990; Bullough et al., 1994). However, it has remained unclear what change is the most critical for the activity.

Various evidence has suggested that a hydrophobic amino acid stretch which is located at the N-terminal of the influenza virus hemagglutinin HA2 chain, called a putative fusion peptide, plays a key role in the controlled interaction of the protein with host cell membranes (Gething et al., 1986; Brunner, 1989; Harter et al., 1989). HA peptide (Figure 1), a 20-residue peptide of the same amino acid sequence as the putative fusion peptide of influenza virus strain A/PR/8/34 hemagglutinin, could induce fusion of phosphatidylcholine liposomes with a similar pH dependence to the protein (Murata et al., 1987a; Lear & DeGrado, 1987). We have studied the fusion activities of a series of synthetic peptides which have amino acid sequences related to a hemagglutinin fusion peptide (Murata et al., 1992). The amino acid sequences of these fusogenic peptides suggested the presence of an amphiphilic secondary structure, α-helix,

HA H-G L F G A I A G F I E G G W Y G M I D G-OH

IV H-G L L E A L A E L L E G G W E G L L E G-OH

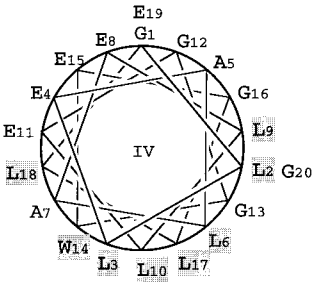

FIGURE 1: Amino acid sequences of HA peptide and peptide IV, and a helical wheel representation of peptide IV. Hydrophobic residues are shaded.

which was confirmed by spectroscopic studies (Takahashi, 1990).

Previously, we reported a study of the orientation of peptide IV (Figure 1), which has the highest α-helical content among related peptides, in egg PC[1] multibilayers by Fourier-transform infrared spectroscopy with an attenuated total reflection (ATR) method (Ishiguro et al., 1993). The result showed that the α-helix axis of the peptide is roughly parallel to the membrane plane (about 70° from the membrane plane normal), and there was no evidence to suggest any orientation changes accompanied by changes of pH or fusion activity of the peptide. However, a system of lipid multibilayers is not appropriate as a model of biomembranes for several reasons. For example, artificial liposomes or biomembranes have single bilayer structures, and not multibilayers. In addition, multibilayers used in the ATR study were prepared in the form of dry or D_2O vapor-hydrated samples, while an actual lipid membrane always makes an interface with the contact solution.

In the present study, we applied the system of supported single planar phospholipid bilayers to IR−ATR spectroscopy,

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[1] Abbreviations: ATR, attenuated total reflections; DOPC, dioleoyl-L-α-phosphatidylcholine; egg PC, egg yolk phosphatidylcholine; IR, infrared; SUV, small unilamellar vesicle(s).

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and studied the process of peptide binding to the bilayers from solution, and the orientation of the peptide at various pHs. The result showed that binding characteristics and orientations of the peptide varied with pH.

MATERIALS AND METHODS

Materials. Peptide IV (Figure 1) was synthesized with a *t*-Boc method on Merrifield's PAM resin (Mitchell et al., 1976; Merrifield et al., 1982). Coupling of amino acids was carried out with their symmetrical anhydrides. Side chains were protected during the synthesis by a benzyl group for glutamic acid, and a formyl group for tryptophan. Cleavage from the resin and deprotection of side chains were achieved with a trifluoromethanesulfonic acid method (Tam et al., 1986). Crude peptide was purified by a reversed-phase HPLC on a COSMOSIL $5C_{18}$-AR column with acetonitrile and ammonium acetate.

Egg PC (egg yolk phosphatidylcholine) and DOPC (di-oleoyl-L-α-phosphatidylcholine) were purchased from Avanti Polar Lipids, and used without further purification.

Preparation of Small Unilamellar Vesicles. Small unilamellar vesicles (SUV) were prepared using a sonication method (Huang, 1969). The final concentration of lipid was determined by Bartlett's method (1959).

Preparation of Supported Single Planar Bilayers of Lipids. Supported single planar bilayers of lipids were prepared on a germanium ATR prism by a combined Langmuir−Blodgett/vesicle−fusion technique (Fringeli, 1989; Frey & Tamm, 1991; Kalb et al., 1992). The ATR prism was thoroughly cleaned with chloroform/methanol (4:1) and by abrasion with air plasma immediately before use, taking the greatest care to avoid residual contamination on the prism.

The first monolayer was prepared on the air/water interface in a Teflon trough with film balance apparatus using a Wilhelmy plate. The orientation of the lipids in the monolayer was achieved by compressing the area of the monolayer to maintain the surface pressure at 32 mN m^{-1}. Under these conditions, the area per one lipid molecule was about 50 $Å^2$; this value should be similar to that occupied by one headgroup of phosphatidylcholine. Then this monolayer was transferred onto the ATR prism as a Langmuir−Blodgett film in an upstroke mode. Using these procedures, we obtained a lipid monolayer on the ATR prism, in which the headgroups of the lipids face toward the prism, and the hydrocarbon chains face outward.

The cell box of the ATR prism with the monolayer was purged with nitrogen to prevent oxidation of unsaturated phosphatidylcholines. Then the prism was soaked in 1 mM SUV suspension (10 mM phosphate and 150 mM KCl, pH 7.5) to induce the fusion of the SUV and the monolayer, and to provide a supported single planar bilayer on the prism. After 5 h, the contact solution was replaced by H_2O, D_2O, and D_2O solutions of 10 mM MES and 150 mM KCl (pD 6.8 or 5.0), in turn. Throughout all operations, careful attention was paid to avoid any contact of the bilayers with air, in order to maintain the regular bilayer structure.

In order to introduce peptide IV into the supported planar bilayers, we equilibrated the bilayer in a D_2O solution (10 mM MES and 150 mM KCl, pD 6.8 or 5.0) of various concentrations of peptide IV.

Soaking the ATR prism in the solutions in order to prepare the supported planar bilayers and to incorporate the peptides on the bilayers could be done without removing the prism on the light path in the IR spectrometer. Thus, it was possible to monitor the fusion of SUV to the lipid monolayer on the prism as an increment of the absorbances of the CH_2-stretching bands, or the binding of the peptides to the planar bilayer as an increment of the absorbance of the amide-I band.

Infrared Spectroscopy. Infrared spectra were recorded on a JASCO FT/IR-7300 spectrometer equipped with an MCT detector, using an apodizing function of a modified cosine curve. During the measurements of spectra, the inner compartment of the spectrometer was purged with air dried over molecular sieves (synthetic zeolite, type A-4, purchased from WAKO Pure Chemical). The resolution was 4 cm^{-1}, and 6400 scans were collected. ATR spectra were taken with a 30°- or 45°-incident germanium prism. Polarized light was obtained with a wire-grid-type polarizer on KRS-5.

The spectra sometimes had to be re-Fourier-transformed to remove the convoluted interference pattern, using software offered by the manufacturer. Other processing of the spectra, such as Fourier self-deconvolution or least-squares curve-fitting, was not used.

Extremely high-sensitivity ATR-mode measurements were required to observe a single layer of lipid molecules or peptides in a single lipid bilayer. Given that lipid or peptide absorption was obtained by subtraction of the reference spectrum (or background) from the sample spectrum, the importance of reference spectra was quite apparent, and a change in the optical path could not be allowed during either measurement. Unfortunately, after the reference spectrum was measured, the ATR prism had to be removed to deposit the lipid film in the Langmuir trough, so a slight change in the optical alignment was unavoidable since we could not reset the lipid-deposited prism at exactly the same position. Even such resetting of the prism did not guarantee a flat base line, and in a control experiment (only resetting the ATR prism without a deposit of lipid film), we found a visible amount of the absorption spectrum not related to the deposited lipid film (some gaseous materials adsorbed somewhere inside the spectrometer were suspected as the origin of this "false" spectrum) and interference fringes. The latter obstruction was eliminated by software supplied by the manufacturer, but as the amount of this "false" spectrum was irreproducible, exact measurement of the absolute spectral intensity of the first lipid monolayer film deposited on the Ge prism was difficult. But once the ATR prism was reset, the following procedures (bilayer formation by liposome fusion onto the lipid monolayer, substitution of an aqueous phase covering the bilayer with solutions of various concentration of peptides, etc.) could be carried out without any perturbation of the optical alignment including the prism, and quality spectra were obtained.

RESULTS

Analysis of Polarized ATR Spectra. Most of the procedure of data analysis of ATR followed Harrick and du Pré (1966) and Harrick (1967). In infrared spectroscopy with an ATR method, the absorbance of material on an ATR prism is determined by the absorption coefficient of the material, relative refractive indices of the sample and prism, the angle of incidence, and the electric field amplitude of the evanescent wave established around the prism surface. In addition,

by using the polarized incident light, we can get information about the anisotropy of the absorption coefficient, from which the average orientation of the materials can be calculated.

The electric field amplitude of the evanescent wave falls off exponentially with distance from the surface. The distance required for the amplitude to fall to e^{-1} of its value at the surface is expressed by d_p, and given by Harrick (1967). In the present experiments, where the thickness of the sample (phospholipid single bilayer, about 70 Å) is much smaller than d_p (estimated as about one-tenth of the wavelength), the absorbance for parallel and perpendicularly polarized incident light can be written as

$$A_p = \frac{Nn_{21}\Gamma(E_x^2 k_x + E_z^2 k_z)}{\cos q}$$

$$A_s = \frac{Nn_{21}\Gamma E_y^2 k_y}{\cos q} \qquad (1)$$

respectively. Subscripts *x*, *y*, and *z* mean the components in the rectangular coordinate system defined as follows: the *z* axis is perpendicular to the prism surface, and the *y* axis is perpendicular to the light path. *N* is the number of internal reflections; *q*, the angle of incidence; $n_{21} = n_2/n_1$, the ratio of the relative refractive indices of the sample film (n_2) and prism (n_1); *k* is the molar absorption coefficient of the spectroscopically detected material in question, and Γ is the surface concentration of the material on the ATR prism, defined as the number of moles per unit surface area of the prism or membrane. *E* is the electric field amplitude of the evanescent wave, which is given as the relative value of the incident light (Harrick & du Pré, 1966; Harrick, 1967), and is different in the case of thin film ($< d_p$, the present case) and of thick film [$> d_p$, (Ishiguro et al., 1993)]. Optical parameters required for calculations were taken from Frey et al. (1991).

According to Brauner et al. (1987), the extinction coefficients of a material are given by

$$k_x = k_y = k_m\left\{\frac{1}{2}f\sin^2 a + \frac{1}{3}(1-f)\right\}$$

$$k_z = k_m\left\{f\cos^2 a + \frac{1}{3}(1-f)\right\} \qquad (2)$$

where *a* is the tilted angle between the molecular axis and the axis of the transition dipole of the absorption in question, and k_m is the molar extinction coefficient when all the transition dipoles are in their optimum orientation. The orientational order parameter *f* is given by

$$f = \frac{3\langle\cos^2\gamma\rangle - 1}{2} \qquad (3)$$

γ is the tilted angle between the molecular axis and the *z* axis.

In order to guess the orientation angle from the *f* value, we calculated γ as an averaged angle by eq 3, assuming that all of the molecules in question take the same orientation. It must be kept in mind that this calculated γ value can be used only qualitatively, though such a procedure has been used in many papers. At the point where $f = 0$ and $\gamma = 54.7°$, called the magic angle, the orientation of molecular axes in question is exactly at the angle of 54.7° or fully isotropic. Larger *f* means that the orientation of the molecules is more perpendicular to the prism surface, and at the point $f = 1$ and $f = -0.5$, all of the molecules have the same tilted angle, which is 0° and 90°, respectively.

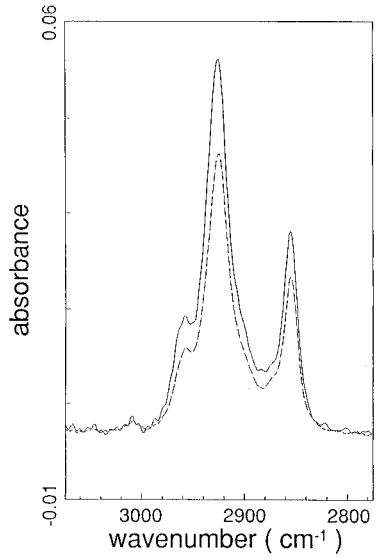

FIGURE 2: IR−ATR spectra of a supported single planar bilayer of egg PC, for parallel (solid curve) and perpendicularly (dashed curve) polarized light.

For the estimation of the orientation of lipids, CH_2-stretching frequencies were used. IR−ATR spectra of the supported planar bilayer of egg PC of this region for parallel and perpendicularly polarized light are shown in Figure 2. From the absorbance at 2926 cm^{-1} (antisymmetric CH_2-stretching) and at 2855 cm^{-1} (symmetric CH_2-stretching) of bilayers and monolayers, we could calculate *f* and γ of the hydrocarbon chains of lipids by eqs 1−3. The tilted angle, *a*, between the transition dipoles of these oscillations and the principal axis of hydrocarbon chains was taken as 90°.

In order to calculate Γ of peptide IV in lipid bilayers and *f* of the α-helix by eqs 1 and 2, we used the peak area of amide-I bands between 1600 and 1700 cm^{-1} as A_p and A_s. These absorbances are determined from the IR−ATR spectra of peptide IV in the lipid bilayer, shown in Figure 3, which were obtained as the difference spectra of the peptide-free and peptide-incorporated bilayers. We took 27° as *a*, the tilted angle between the α-helix axis and the transition dipole of peptide groups (Rothschild & Clark, 1979).

We estimated k_m of peptide IV from the absorbance of the peptide in a KBr tablet. From the slope ($=k_m/3$) of a plot of absorbance area vs the number of peptide moles per tablet surface, the value was determined as 501 μmol^{-1} cm^2.

The peak height of amide-I was too small to be decomposed into the component peaks of each secondary structure spectrum. We reported previously that, according to a CD spectral study, peptide IV mainly assumed α-helix in the presence of lipid vesicles (Takahashi, 1990). We assumed that peptide IV adopted α-helix in single bilayers, because the wavenumber of the peak of the amide-I falls in the range of 1645−1650 cm^{-1}, suggesting this secondary structure was dominant.

Supported Single Planar Bilayers and Monolayers. Evidence for the formation of a supported lipid monolayer or bilayer on an ATR prism was obtained by infrared spectra. Although results of spectral intensity measurements of the first lipid layer (Langmuir−Blodgett monolayer) on an ATR

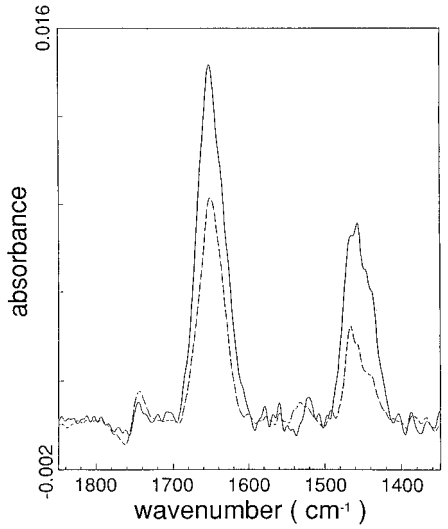

FIGURE 3: IR−ATR spectra of peptide IV in a supported single planar bilayer of egg PC equilibrated in D_2O solution of 0.33 μM peptide IV, 10 mM MES, and 150 mM KCl at pD 5, for parallel (solid curve) and perpendicularly (dashed curve) polarized light. These were obtained as the differences between the spectra of the peptide-free and peptide-incorporated bilayers.

Table 1: Orientations of Lipids in Supported Single Planar Bilayers and Monolayers[a]

	$\nu_{as}(CH_2)$[b] (2926 cm^{-1})		$\nu_s(CH_2)$[c] (2855 cm^{-1})	
	f[d]	γ[e]	f	γ
DOPC bilayer	0.03 ± 0.11	54 ± 4	0.09 ± 0.15	51 ± 6
DOPC monolayer	0.22 ± 0.08	46 ± 3	0.27 ± 0.07	45 ± 3
egg PC bilayer	0.14 ± 0.03	49 ± 1	0.19 ± 0.04	47 ± 2
egg PC monolayer	0.39 ± 0.16	40 ± 6	0.27 ± 0.07	45 ± 3

[a] Deviations show the upper and lower limits of the values obtained from multiple experiments. [b] Antisymmetric CH_2-stretching band. [c] Symmetric CH_2-stretching band. [d] Order parameter of the hydrocarbon chains. [e] The tilted angle of the principal molecular axis of hydrocarbon chains from the membrane normal.

prism were ambiguous, the increment of absorbance over the whole spectral range upon the formation of the second lipid layer by liposome fusion was always at a similar level, irrespective of lipid species. Since the procedure of deposition of a lipid film according to the Langmuir−Blodgett method is well established, we believe the first layer is a lipid monolayer. We considered that the fact that the absorption intensity was nearly doubled upon liposome fusion onto the monolayer evidenced the completion of lipid bilayer formation.

Orientational parameters f and γ of hydrocarbon chains of egg PC and DOPC in single bilayers and monolayers are summarized in Table 1. Although the reliability was slightly questioned due to a large degree of mean deviation (víde ínfra), the result was apparently contradictory to those reported previously by some researchers. A value of about 0.4 for f and γ of about 40° had been reported for hydrocarbon chains on liposomes in liquid-crystalline states from NMR and ESR studies (Marcelja, 1974; Seelig & Seelig, 1977), although nearly random orientation (0−0.1 for f) in planar multibilayers had also been reported from an IR−ATR study (Fringeli et al., 1976). However, the orientation in supported single bilayers in liquid-crystalline states has not been reported. These discrepancies may come from the differences between the experimental systems. It should be mentioned that, as described under Materials and

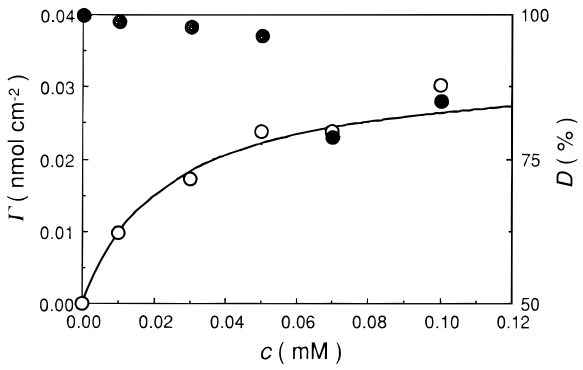

FIGURE 4: Binding isotherm of peptide IV to the egg PC bilayer from the neutral D_2O solution (10 mM MES and 150 mM KCl, pD 6.8), and influence on the lipid bilayer. Γ (nmol cm^{-2}) (open circles) is the surface concentration of the peptides in the lipid bilayer, and c (mM) is the peptide concentration of the contact solution equilibrated with the bilayer. The solid curve is a Langmuir's binding isotherm fitted to the experimental data. D (%) (closed circles) is the relative value of the absorbance area of lipid C═O stretching (1690−1770 cm^{-1}) to that of the peptide-free single bilayer.

Methods, there was some ambiguity in determining the absolute absorbance of the first lipid layer in the present measurements, which yielded a considerable amount of the mean deviation, and might also influence the obtained f values. Considering this limitation of the spectroscopy with respect to the orientation of the hydrocarbon chains of phospholipids, only the differences between f values in the peptide-free single bilayers and those in the presence of the peptide will be discussed hereafter.

Binding Isotherms of the Peptides to the Bilayers. Figure 4 shows the binding isotherm of peptide IV to the egg PC bilayer from the neutral D_2O solution and the influence of peptide binding on the lipid. The experimental data can be fitted with a Langmuir's adsorption isotherm:

$$\Gamma = \frac{K\Gamma_m c}{1 + Kc} \tag{4}$$

by assuming that the binding constant, K, is 42.5 mM^{-1} and the maximum value of Γ, Γ_m, is 0.0325 nmol cm^{-2}. These two parameters are determined by applying a least-squares fitting of the plot of the experimental data of c/Γ vs c.

The number of egg PC molecules in the bilayer begins to decrease near $\Gamma = \Gamma_m$, which is detected as the decrease of the absorbance of the lipid C═O stretching, and nearly 50% of the lipid molecules of the outer monolayer are estimated to vanish from the bilayer. This result was thought to be due to the replacement or solubilization of lipid molecules with peptide IV. If we construct the α-helix model taking the side chains' bulkiness into consideration, peptide IV can be thought of as a cylinder with a radius of 5 Å and a height of 30 Å, and so the peptide monomer is assumed to occupy 80−300 Å^2 of the surface, dependent on its orientation and depth. Thus, at $\Gamma = \Gamma_m$, the peptides can replace about 17−65% of the membrane surface.

As for the acidic D_2O contact solution (Figure 5), a binding isotherm to egg PC or DOPC bilayers shows completely different characteristics. Binding isotherms appear to be sigmoidal, suggesting that the binding is cooperative, and the replacement of lipids with peptides was not observed, in contrast to the case with neutral pH. Supposing that peptide IV exists as a monomer in the contact solution, is

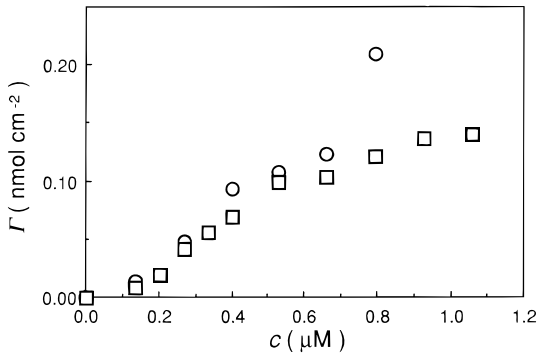

FIGURE 5: Binding isotherms of peptide IV to the lipid bilayers from the acidic D_2O solution (10 mM MES and 150 mM KCl, pD 5). Circles, DOPC; squares, egg PC.

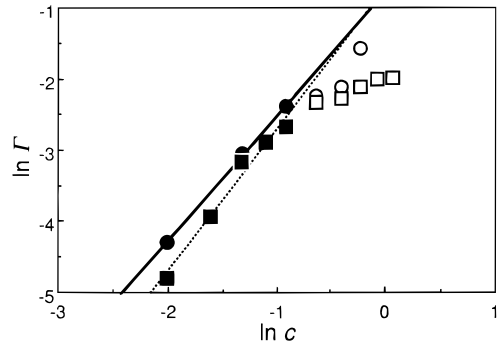

FIGURE 6: Logarithmic plot of the data in Figure 5. Circles, DOPC; squares, egg PC. Solid and dotted lines are calculated by a least-squares fitting from the experimental data at low Γ (closed symbols), and their slopes are 1.7 and 2.1, respectively.

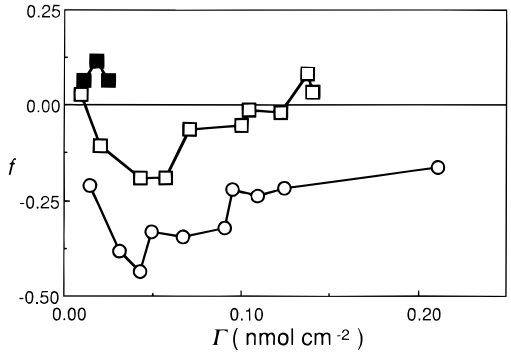

FIGURE 7: Surface concentration dependency of the orientation of peptide IV in lipid bilayers. Γ is the surface concentration of the peptides in lipid bilayers. *f* is the order parameter of the α-helix axis. Circles, DOPC; squares, egg PC; open symbols, pD 5; closed symbols, pD 6.8.

bound to the lipid bilayers, and is then associated to *n*-mer, the binding isotherm at low Γ is expressed as

$$\ln \Gamma = \ln (nK_n) + n \ln (K\Gamma_m) + n \ln c \qquad (5)$$

where K_n is the association constant of the peptides in the bilayer. Figure 6 is a logarithmic plot of the data in Figure 5. The slopes, *n*, are 1.7 and 2.1, respectively for DOPC and egg PC, suggesting that the association number is 2 and that the incorporation of peptide IV into lipid bilayers is cooperative.

Orientation of the α-Helix Axis. The results on *f* of peptide IV α-helix at various Γ of the peptide are summarized in Figure 7. In the binding from the neutral contact solution to egg PC bilayers, α-helix axes are nearly random or have

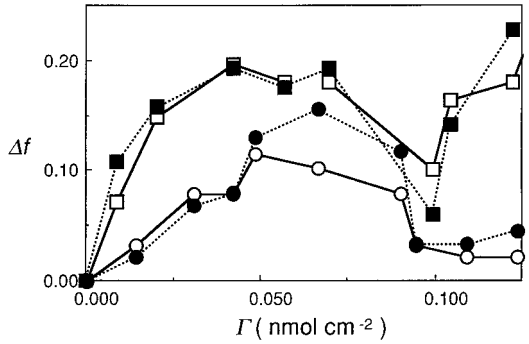

FIGURE 8: Influence of peptide IV on the orientation of the lipids. Δ*f* means a change in the order parameter of the hydrocarbon chain calculated from the absorbances of the antisymmetric CH_2-stretching band (open symbols) and the symmetric CH_2-stretching band (closed symbols). Γ is the surface concentration of the peptides in lipid bilayers. Circles, DOPC; squares, egg PC.

about 54.7° orientation from the membrane normal. As for the acidic contact solution, α-helix axis orientations in egg PC bilayers are observed to be relatively parallel to the membrane surface, and the orientations in DOPC bilayers are even more parallel (as a mean) to the membrane surface than in egg PC bilayers. At low Γ, the orientations become more parallel to the membrane surface as binding proceeds. But at the points where Γ is over 0.05 nmol cm^{-2}, the orientation abruptly changes to more random or more perpendicular to the membrane surface. Assuming that the parallel peptide monomer occupied about 300 $Å^2$ of the membrane surface, 0.05 nmol cm^{-2} is estimated as the maximum Γ. Thus, a change of orientation must be required for further binding.

Influence of Peptide IV on Lipid Bilayers. Figure 8 shows the influence of peptide binding on the order parameter *f* of the hydrocarbon chains of the lipids. The hydrocarbon chains are oriented more perpendicularly to the membrane surface with the peptide binding in both egg PC or DOPC bilayers, as revealed by the analysis of the data on both antisymmetric CH_2-stretching and symmetric CH_2-stretching absorbances. The mean tilted angle of the hydrocarbon chains, γ, reached a minimum at the nearly maximum parallel peptide binding ($\Gamma = 0.05$ nmol cm^{-2}). These results suggest that the surface pressure or the surface energy of the lipid bilayers increases, probably owing to the insertions of the hydrophobic side chains of peptide IV into the bilayers, and the insertions occur more effectively when the peptides are parallel to the membrane surface.

DISCUSSION

The purpose of this paper is to show the behavior of a synthetic fusogenic peptide related to the putative fusion peptide in influenza hemagglutinin in supported single planar phosphatidylcholine bilayers, especially in comparison with the result obtained for supported multibilayers. We are also interested in the possible relevance of the process of virus-induced membrane fusion, although there are substantial differences between virus-induced and peptide-induced fusion.

Peptide IV and related peptides induce membrane fusion not at neutral pH but at acidic pH (Murata et al., 1987a, 1992). Nevertheless, peptide IV interacts with liposomes assuming α-helical structures both at neutral and at acidic pH (Takahashi, 1990). Thus, the first trigger inducing

membrane fusion was expected to be protonation of the carboxylate groups in the hydrophilic side of amphiphilic α-helices (Murata et al., 1987b). It is possible that such protonation, resulting in an increase in hydrophobicity of the residues, induces a change of peptide orientation, which may be in turn related to triggering the fusion. Thus, we have tried to determine the orientation of the α-helices in lipid membranes at acidic and neutral pH, which must reflect the behavior of the peptides.

The orientations of secondary structures of various groups of peptides, such as opioid peptides, hormones, biosurfactants and signal peptides in lipid membranes have been studied using IR−ATR spectroscopy (Gremlich et al., 1983; Erne et al., 1985; Schwyzer, 1986; Cornell et al., 1989; Pastrana et al., 1991; Vandenbussche et al., 1992; Bauer et al., 1994). Several putative transmembrane α-helical peptide fragments of membrane proteins have been confirmed to be actually perpendicular to the membrane surface (Smith, S. O., et al., 1994; Arkin et al., 1995; Tatulian et al., 1995; Zhang et al., 1995).

The orientation of the α-helical axis of peptide IV on supported single planar bilayers depended on pH, and on the surface concentration of the bound peptide. As far as pH dependency is concerned, the orientation tended to be parallel to the membrane surface at acidic pH, and to be random or oblique at neutral pH (Figure 7). Roughly parallel orientation of the peptide to the membrane surface at acidic pH was also suggested by hydrophobic photolabeling studies of the fusion peptide in influenza hemagglutinin (Brunner, 1989; Harter et al., 1989) and by our previous paper, in which we determined the orientation of α-helical axes of the synthetic fusogenic peptides in egg PC multibilayers by IR−ATR spectroscopy (Ishiguro et al., 1993). This contrasts with the oblique or membrane-penetrating orientation deduced for the synthetic α-helical peptides corresponding to the fusion peptides of other viral fusion proteins, gp41 of HIV, and gp32 of SIV in lipid multibilayers (Martin, 1991, 1993a,b, 1994). Peptide IV has a more uniform disposition of hydrophobic and hydrophilic residues along the helix axis and a stronger amphiphilicity than those studied by Martin et al., and these characteristics are thought to permit the orientation of peptide IV parallel to the membrane surface at acidic pH. At neutral pH, the peptide does not take parallel orientation any longer, in spite of the larger amphiphilicity. This suggests that the peptides are bound to the membrane at a shallower level of the inner hydrophobic than they are at acidic pH.

Slight discrepancies were found between our present and previous results. When the surface concentration of the peptide was about 0.01 nmol cm^{-2} (corresponding to a lipid/peptide mole ratio of about 20), the α-helix axis orientation in the single bilayer of egg PC was nearly random or oblique (about 54.7° from the membrane normal) at both acidic and neutral pH (Figure 7) in the present result, while in dry or D_2O-hydrated multibilayers in the previous study it was more parallel to the membrane surface (about 70° from the membrane normal) (Ishiguro et al., 1993). We attribute these discrepancies to the differences between the environments, *i.e.*, a supported single bilayer and multibilayers. We considered that a supported single planar bilayer was more suitable for the model of a lipid membrane than multibilayers, as discussed in the previous section of this paper. Furthermore, we could control the pH or the composition of the contact solution more rigorously, and could simultaneously observe the process of the binding of the peptide and their orientation in single bilayers.

With respect to melittin, which is a membrane-lytic peptide isolated from bee venom and a 26-residue amphiphilic α-helical peptide with a bent conformation, and alamethicin, a voltage-gated channel-forming peptide which is therefore considered to be a membrane-spanning α-helix and to aggregate to form a pore, it has also been reported that the orientation of α-helices in lipid bilayers depends on the experimental conditions. Their α-helix axes in dry multibilayers have been shown to be perpendicular to the membrane surface in studies using CD, NMR, and IR−ATR spectroscopy (Fringeli & Fringeli, 1979; Brauner et al., 1987; Vogel, 1987; Weaver et al., 1992; de Jongh, 1994; Smith, R., et al., 1994). On the other hand, the α-helix axis of melittin in supported single planar bilayers and liposomes exhibits parallel orientation to the membrane surface according to studies with IR−ATR spectroscopy (Frey & Tamm, 1991), and amide hydrogen−deuterium exchange experiments using NMR spectra (Dempsey & Butler, 1992). Vogel (1987) has shown that the orientation of melittin and alamethicin in lipid multibilayers depends largely on the extent of hydration of the membranes and on temperature. Moreover, Fringeli et al. (1979) have shown that the hydration influences even the secondary structure of alamethicin.

Regarding the second point of peptide binding to supported single planar bilayers, two aspects were considered: cooperativity in peptide binding, and the dependence of peptide orientation on peptide surface concentration in membranes. At acidic pH, peptide IV was bound to single bilayers in a cooperative manner, which we ascribed to the association of the peptides in the membrane (Figure 5), while binding at neutral pH appears to be a noncooperative Langmuir's adsorption isotherm (Figure 4). Cooperative binding has also been observed in alamethicin and magainin, both of which are channel-forming antimicrobial peptides, by titration studies using IR−ATR and fluorescence spectroscopy (Fringeli, 1980; Matsuzaki et al., 1994).

The dependence of peptide orientation on the surface concentration of the peptide in the bilayer (Figure 7) might be explained from the viewpoint of supported single bilayers as follows. Peptide IV has a tendency to be oriented parallel to the membrane surface owing to the amphiphilic character of the peptide. In supported single planar bilayers, lipid fluidity observed in vesicles is well preserved (Kalb et al., 1992). So at low surface concentrations of the peptide, the order of the peptides is disturbed by the motion of the lipid molecules, causing the relatively random orientation of α-helices. As more of the peptides bind at acidic pH, the fluidity is suppressed by the increment of surface energy or surface pressure (Figure 8, seen as an increase in the order parameter of lipid hydrocarbon chains), and the peptides can take up their inherent parallel orientation. In the case of egg PC, a more random orientation of the peptide was expected due to its heterogeneous lipid compositions, unlike DOPC, and was actually observed. Beyond the point of maximum parallel binding, the peptides would not be able to take the optimum parallel orientation for further binding, and would not influence the order of lipids any longer, and there may be segregation of the peptide (Figure 8).

On the other hand, at neutral pH, the peptides are bound to lipid bilayers in the manner of a noncooperative Langmuir's adsorption isotherm (Figure 4), suggesting that the peptide exists in the same aggregation state both in solution and in lipid bilayers. Since the peptide has the activity of a surfactant because of charges in the hydrophilic side, excess binding may induce the replacement or solubilization of lipid molecules, and the study of the orientation of the peptides over the range has no meaning.

How does the fusion peptide behave in influenza hemagglutinin in an active state or how does it behave as a synthetic peptide? Acidic pH causes a conformational change in hemagglutinin and the binding to the target membrane by the exposed fusion peptide (Gething et al., 1986; Brunner, 1989; Harter et al., 1989). At the same time, the hydrophobicity of the peptides may destabilize the outer monolayer of the membrane. In view of the structure of single bilayers, in which the hydrocarbon chain layer is separated from the aqueous layer by hydrophilic headgroups of phosphatidylcholine, destabilizing the outer monolayer is enough to destabilize the whole bilayer structure. Subsequently, some fusogenic machinery may be used to complete membrane fusion (Morris et al., 1889; Spruce et al., 1989, 1991; Ellens et al., 1990; White, 1992). In the case of the synthetic peptide, on the other hand, destabilizing the surface of liposomes and dimerization of the peptides on the different liposomes may cause the aggregation of liposomes.

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REFERENCES

Arkin, I. T., Rothman, M., Ludlam, C. F. C., Aimoto, S., Engelman, D. M., Rothschild, K. J., & Smith, S. O. (1995) *J. Mol. Biol. 248*, 824−834.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466−468.

Bauer, H. H., Müller, M., Goette, J., Merkle, H. P., & Fringeli, U. P. (1994) *Biochemistry 33*, 12276−12282.

Brauner, J. W., Mendelsohn, R., & Prendergast, F. G. (1987) *Biochemistry 26*, 8151−8158.

Brunner, J. (1989) *FEBS Lett. 257*, 369−372.

Bullough, P. A., Hughson, F. M., Skehel, J. J., & Wiley, D. C. (1994) *Nature 371*, 37−43.

Cornell, D. G., Dluhy, R. A., Briggs, M. S., McKnight, J., & Gierasch, L. M. (1989) *Biochemistry 28*, 2789−2797.

de Jongh, H. H. J., Goormaghtigh, E., & Killian, J. A. (1994) *Biochemistry 33*, 14521−14528.

Dempsey, C. E., & Butler, G. S. (1992) *Biochemistry 31*, 11973−11977.

Doms, R. W., Helenius, A., & White, J. (1985) *J. Biol. Chem. 260*, 2973−2981.

Doms, R. W., Gething, M.-J., Henneberry, J., White, J., & Helenius, A. (1986) *J. Virol. 57*, 603−613.

Ellens, H., Bentz, J., Mason, D., Zhang, F., & White, J. M. (1990) *Biochemistry 29*, 9697−9707.

Erne, D., Sargent, D. F., & Schwyzer, R. (1985) *Biochemistry 24*, 4261−4263.

Frey, S., & Tamm, L. K. (1991) *Biophys. J. 60*, 922−930.

Fringeli, U. P. (1980) *J. Membr. Biol. 54*, 203−212.

Fringeli, U. P. (1989) in *Biologically Active Molecules* (Schlunegger, U. P., Ed.) pp 241−252, Springer-Verlag, Berlin.

Fringeli, U. P., & Fringeli, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3852−3856.

Fringeli, U. P., Schadt, M., Rihak, P., & Günthard, Hs. H. (1976) *Z. Naturforsch. 31A*, 1098−1107.

Gething, M.-J., Doms, R. W., York, D., & White, J. (1986) *J. Cell Biol. 102*, 11−23.

Gremlich, H.-U., Fringeli, U. P., & Schwyzer, R. (1983) *Biochemistry 22*, 4257−4264.

Harrick, N. J. (1967) *Internal Reflection Spectroscopy*, Interscience Publishers, New York.

Harrick, N. J., & du Pré, F. K. (1966) *Appl. Opt. 5*, 1739−1743.

Harter, C., James, P., Bächi, T., Semenza, G., & Brunner, J. (1989) *J. Biol. Chem. 264*, 6459−6464.

Huang, C. (1969) *Biochemistry 8*, 344−352.

Ishiguro, R., Kimura, N., & Takahashi, S. (1993) *Biochemistry 32*, 9792−9797.

Kalb, E., Frey, S., & Tamm, L. K. (1992) *Biochim. Biophys. Acta 1103*, 307−316.

Lear, J. D., & DeGrado, W. F. (1987) *J. Biol. Chem. 262*, 6500−6505.

Maeda, T., & Ohnishi, S. (1980) *FEBS Lett. 122*, 283−287.

Maeda, T., Kawasaki, K., & Ohnishi, S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4133−4137.

Marčelja, S. (1974) *Biochim. Biophys. Acta 367*, 165−176.

Martin, I., Defrise-Quertain, F., Mandieau, V., Nielsen, N. M., Saermark, T., Burny, A., Brasseur, R., Ruysschaert, J.-M., & Vandenbranden, M. (1991) *Biochem. Biophys. Res. Commun. 175*, 872−879.

Martin, I., Defrise-Quertain, F., Decroly, E., Vandenbranden, M., Brasseur, R., & Ruysschaert, J.-M. (1993a) *Biochim. Biophys. Acta 1145*, 124−133.

Martin, I., Dubois, M.-C., Saermark, T., Epand, R. M., & Ruysschaert, J.-M. (1993b) *FEBS Lett. 333*, 325−330.

Martin, I., Dubois, M.-C., Defrise-Quertain, F., Saermark, T., Burny, A., Brasseur, R., & Ruysschaert, J.-M. (1994) *J. Virol. 68*, 1139−1148.

Matsuzaki, K., Murase, O., Tokuda, H., Funakoshi, S., Fujii, N., & Miyajima, K. (1994) *Biochemistry 33*, 3342−3349.

Merrifield, R. B., Vizioli, L. D., & Boman, H. G. (1982) *Biochemistry 21*, 5020−5031.

Mitchell, A. R., Erickson, B. W., Ryabstev, M. N., Hodges, R. S., & Merrifield, R. B. (1976) *J. Am. Chem. Soc. 98*, 7357−7362.

Morris, S. J., Sarkar, D. P., White, J. M., & Blumenthal, R. (1989) *J. Biol. Chem. 264*, 3972−3978.

Murata, M., Sugahara, Y., Takahashi, S., & Ohnishi, S. (1987a) *J. Biochem. 102*, 957−962.

Murata, M., Nagayama, K., & Ohnishi, S. (1987b) *Biochemistry 26*, 4056−4062.

Murata, M., Takahashi, S., Kagiwada, S., Suzuki, A., & Ohnishi, S. (1992) *Biochemistry 31*, 1986−1992.

Pastrana, B., Mautone, A. J., & Mendelsohn, R. (1991) *Biochemistry 30*, 10058−10064.

Rothschild, K. J., & Clark, N. A. (1979) *Biophys. J. 25*, 473−488.

Ruigrok, R. W. H., Wrigley, N. G., Calder, L. J., Cusack, S., Wharton, S. A., Brown, E. B., & Skehel, J. J. (1986) *EMBO J. 5*, 41−49.

Schwyzer, R. (1986) *Biochemistry 25*, 4281−4286.

Seelig, A., & Seelig, J. (1977) *Biochemistry 16*, 45−50.

Skehel, J. J., Bayley, P. M., Brown, E. B., Martin, S. R., Waterfield, M. D., White, J. M., Wilson, I. A., & Wiley, D. C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 968−972.

Smith, R., Separovic, F., Milne, T. J., Whittaker, A., Bennett, F. M., Cornell, B. A., & Makriyannis, A. (1994) *J. Mol. Biol. 241*, 456−466.

Smith, S. O., Jonas, R., Braiman, M., & Bormann, B. J. (1994) *Biochemistry 33*, 6334−6341.

Spruce, A. E., Iwata, A., White, J. M., & Almers, W. (1989) *Nature 342*, 555−558.

Spruce, A. E., Iwata, A., & Almers, W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3623−3627.

Stegmann, T., Doms, R. W., & Helenius, A. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 187−211.

Stegmann, T., White, J. M., & Helenius, A. (1990) *EMBO J. 9*, 4231−4241.

Takahashi, S. (1990) *Biochemistry 29*, 6257−6264.

Tam, J. P., Heath, W. F., & Merrifield, R. B. (1986) *J. Am. Chem. Soc. 108*, 5242−5251.

Tatulian, S. A., Jones, L. R., Reddy, L. G., Stokes, D. L., & Tamm, L. K. (1995) *Biochemistry 34*, 4448−4456.
Vandenbussche, G., Clercx, A., Clercx, M., Curstedt, T., Johansson, J., Jörnvall, H., & Ruysschaert, J.-M. (1992) *Biochemistry 31*, 9169−9176.
Vogel, H. (1987) *Biochemistry 26*, 4562−4572.
Weaver, A. J., Kemple, M. D., Brauner, J. W., Mendelsohn, R., & Prendergast, F. G. (1992) *Biochemistry 31*, 1301−1313.
Wharton, S. A., Ruigrok, R. W. H., Martin, S. R., Skehel, J. J., Bayley, P. M., Weis, W., & Wiley, D. C. (1988) *J. Biol. Chem. 263*, 4474−4480.
White, J. M. (1992) *Science 258*, 917−924.
White, J. M., & Wilson, I. A. (1987) *J. Cell Biol. 105*, 2887−2896.
White, J., Kielian, M., & Helenius, A. (1983) *Q. Rev. Biophys. 16*, 151−195.
Zhang, Y.-P., Lewis, R. N. A. H., Henry, G. D., Sykes, B. D., Hodges, R. S., & McElhaney, R. N. (1995) *Biochemistry 34*, 2348−2361.
Zimmerberg, J., Vogel, S. S., & Chernomordik, L. V. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 433−466.

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