Hypelcin A, an α-Aminoisobutyric Acid Containing Antibiotic Peptide, Induced Permeability Change of Phosphatidylcholine Bilayers

Katsumi Matsuzaki,‡ Shunji Nakai,‡ Tetsurou Handa,‡ Yoshihisa Takaishi,§ Teturo Fujita,‡ and Koichiro Miyajima*,‡

Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606 Japan, and Faculty of Pharmaceutical Sciences, Tokushima University, Sho-machi, Tokushima, 770 Japan

Received March 1, 1989; Revised Manuscript Received July 20, 1989

ABSTRACT: Interactions of hypelcin A, an α-aminoisobutyric acid containing antibiotic peptide, with phosphatidylcholine vesicles were investigated to obtain information on its bioactive mechanism. The peptide induced the leakage of a fluorescent dye, calcein, entrapped in sonicated vesicles. The leakage rate depended on both the peptide and the lipid concentrations. Analysis of this dependency indicated that the leakage was due to the monomeric peptide and that the membrane-perturbing activity of the monomer was higher for solid distearoylphosphatidylcholine vesicles than for fluid egg yolk phosphatidylcholine vesicles. Hypelcin A also affected the gel to liquid-crystalline phase transition of dipalmitoylphosphatidylcholine multilamellar vesicles. The transition was broadened with a reduced transition enthalpy, suggesting the peptide strongly binds the surrounding lipids to perturb the bilayer lipid packing. A circular dichroism study revealed that the helical content of hypelcin A increases upon membrane binding. We concluded that the monomeric peptide with an increased helical content, complexed with the lipids, perturbs the lipid organization and induces the increased permeability.

Hypelcin A, isolated from *Hypocrea peltata*, is a hydrophobic peptide containing α-aminoisobutyric acid (Aib)[1] residues (Fujita et al., 1984a, 1979). The peptide is a mixture of at least four peptides with similar amino acid sequences. The two major components, hypelcins A-I and A-II, have the following sequences: Ac-Aib-Pro-Aib-Ala-Aib^5-Aib(for A-I; Ala for A-II)-Gln-Aib-Leu-Aib^{10}-Gly-Aib-Aib-Pro-Val^{15}-Aib-Aib-Gln-Gln-leucinol. Hypelcin A inhibits the growth of various fungi and bacteria (Fujita et al., 1984b, 1979) and uncouples oxidative phosphorylation in rat liver mitochondria (Takaishi et al., 1980). These bioactivities are common features in Aib-containing peptides, such as alamethicin, antiamoebin, and suzukacillin, which also show other membrane-modifying properties, that is, the formation of voltage-gated ion channels, hemolysis, and membrane fusion (Boheim et al., 1976; Das et al., 1986; Eisenberg et al., 1973; Irmscher & Jung, 1977; Lau & Chan, 1974; Mathew et al., 1982). However, the detailed mechanisms of the above activities are still veiled in spite of extensive studies. The investigation of the interactions of hypelcin A with lipid vesicles, a simple membrane system, will give us some basic information on the molecular machinery for the activities.

Peptide–lipid interactions have been studied not only to reveal the action mechanisms of bioactive peptides but also to understand the organizational principles of protein–lipid molecular assemblies, such as biomembranes and lipoproteins, or the biological processes involving peptide–lipid interactions, for example, virus infection and the secretion of biosynthesized proteins. Many amphiphilic or hydrophobic peptides, both natural and synthetic, are known to interact with lipid membranes, causing changes in the physicochemical properties of the membranes (e.g., the permeability and the phase transition) or inducing the fusion of the membranes. These interactions have been discussed in terms of conformational changes of both the peptide and the lipid molecules detected by various spectroscopic and thermal techniques (Banerjee et al., 1985; Epand et al., 1983; Kubesch et al., 1987; Subbarao et al., 1987).

Thus, as the first step to understanding the entire features of the hypelcin A–lipid interactions, we studied the effects of hypelcin A on the permeability of lipid vesicles in the gel state (distearoylphosphatidylcholine) and in the liquid-crystalline state (egg yolk phosphatidylcholine) as evaluated by the leakage of an entrapped fluorescent dye, calcein (Allen & Cleland, 1980). This approach, although popular in studying peptide–lipid interactions, has hardly been adopted for the Aib-containing peptides probably because interest has been mainly focused on the voltage-gated ion channels in planer lipid bilayers. The consideration of the leakage mechanism will be served by two sets of data on (1) the binding isotherm of the peptide to the membranes and (2) the relationship between the amount of the membrane-bound peptide and the permeability change (Matsuzaki et al., 1988, 1989; Rizzo et al., 1987). The former informs us of the intramembrane aggregational states of the peptide whereas the latter of the "membrane-perturbing activity" of the peptide. These data can be obtained by analyzing both the peptide concentration and the lipid concentration dependencies of the leakage rate. Furthermore, we examined the effects of hypelcin A on the gel to liquid-crystalline phase transition of dipalmitoylphosphatidylcholine bilayers by using a differential scanning calorimetry (DSC) technique. The phase transition reflects the modification of the lipid–lipid interactions. Conformational

‡ Kyoto University.
§ Tokushima University.

[1] Abbreviations: Aib, α-aminoisobutyric acid; DSC, differential scanning calorimetry; CD, circular dichroism; egg PC, egg yolk L-α-phosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; DSPC, L-α-distearoylphosphatidylcholine; SA, stearylamine; NRMSD, normalized root mean standard deviation; REV, reverse-phase evaporation vesicle.

0006-2960/89/0428-9392$01.50/0

changes of the peptide upon binding to the membranes were examined with its circular dichroism (CD) spectra. We will discuss hypelcin A–lipid interactions on the basis of these data.

Materials and Methods

Materials. Hypelcin A was isolated from *Hypocrea peltata* as described previously (Fujita et al., 1984b, 1979) and stored in methanol at 4 °C. The peptide concentration was based on quantitative amino acid analysis (Fujita et al., 1984b): One mole of hypelcin A (a mean molecular weight of 1927 was assumed) contained 1.01 mol of Val, 1.02 mol of Gly, 2.06 mol of Pro, and 3.03 mol of Gln, guaranteeing a high purity of the peptide. These amino acids occur commonly in the four microheterogeneous components. Egg yolk L-α-phosphatidylcholine (egg PC), L-α-dipalmitoylphosphatidylcholine (DPPC), L-α-distearoylphosphatidylcholine (DSPC), stearylamine (SA), and gramicidin were purchased from Sigma. Calcein (3,3′-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-fluorescein) and spectrograde organic solvents were supplied by Dojin (Kumamoto, Japan). Ammonium *d*-camphor-10-sulfonate was a product of Katayama (Osaka, Japan). Poly(L-lysine) hydrobromide (mean molecular weight was 56 600) was provided by Miles-Yeda. All other chemicals were obtained from Wako (Tokyo, Japan). A 10mM Tris-HCl/150 mM NaCl buffer (pH 7.0) was prepared with water twice distilled from a quartz still.

Leakage from Sonicated Vesicles. Calcein-entrapped sonicated vesicles of egg PC or DSPC were prepared as follows. Aliquots of a lipid solution in chloroform/methanol were placed in a round-bottom 20-mL flask. After evaporation of the solvent, the residual film was dried under vacuum overnight. The lipid film was hydrated with a 70 mM calcein solution (pH was adjusted to 7.0 with NaOH). The suspension was vortexed, followed by sonication in ice–water with nitrogen bubbling for 20 min by using a titanium tip sonicator (Tomy UD-200). In the preparation of DSPC vesicles, the hydration and the sonication were carried out at 60 °C. Metal debris from the tip was removed by centrifusion. Untrapped calcein was removed by gel filtration (Sephadex G-50, 2 × 35 cm column, the buffer being used as an eluent). The separated vesicular fraction was appropriately diluted. The vesicles were mixed with calcein-free sonicated vesicles to obtain the desired lipid concentration. The lipid concentration was determined by phosphorus analysis (Bartlett, 1959). Quasi-elastic light scattering measurements (Otsuka Electronics, Model DLS-700) of the vesicles showed a number-averaged diameter of ca. 25 nm, typical of small unilamellar vesicles. Small aliquots of a 200 μM hypelcin A/methanol solution were added into 3 mL of the vesicular suspension under stirring in a thermostated (30 ± 0.5 °C) quartz cuvette. The methanol concentration was less than 2.5% v/v. The leakage of calcein from the vesicles was monitored fluorometrically (excitation at 490 nm and emission at 520 nm; Allen & Cleland, 1980). The fluorescence intensity corresponding to 100% leakage was determined by addition of 150 μL of a 10% v/v Triton X-100 solution. Percent leakage was calculated after volume correction for dilution.

Leakage from Reverse-Phase Evaporation Vesicles (REVs). The REVs of egg PC were prepared as described previously (Szoka & Papahadjopoulos, 1978) in a 10 mM Tris-HCl/150 mM KCl buffer (pH 7.0) or the 70 mM calcein solution. The vesicles were gel filtrated (Sephadex G-50) with the Tris/NaCl buffer as an eluent. A gramicidin/methanol solution was added to the K^+- or the calcein-loaded vesicles. The final concentrations of gramicidin and egg PC were 4 and 700 μM, respectively. The leakage of K^+ was monitored by a K^+-selective electrode (Orion Research, Model 93-19) with a double-junction reference electrode (Model 90-02) connected to a Model 901 microprocessor ion analyzer. The K^+ concentration corresponding to 100% leakage was determined by addition of a 10% v/v Triton X-100 solution. The calcein leakage was detected as previously described. The temperature was maintained at 30 ± 0.5 °C.

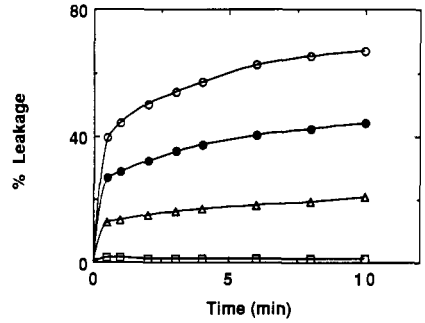

FIGURE 1: Time course of hypelcin A induced calcein leakage from egg PC sonicated vesicles. Small aliquots of a hypelcin A/methanol solution were added to calcein-loaded egg PC sonicated vesicles at 30 °C. The leakage of the dye was monitored fluorometrically. Experimental conditions ($[P]_0$, the peptide concentration in μM; [L], the lipid concentration in μM): open circles, $[P]_0 = 3$ and $[L] = 175$; closed circles, $[P]_0 = 3$ and $[L] = 273$; triangles, $[P]_0 = 2.3$ and $[L] = 175$; squares, $[P]_0 = 0$ and $[L] = 175$.

DSC. Aliquots of a hypelcin A/methanol solution and a DPPC/chloroform solution were mixed in a test tube. After evaporation of the solvent, the residual film was dried under vacuum overnight. The buffer was added onto the film, and the suspension was vortexed at 60 °C. The vesicles thus prepared were cooled to room temperature and then reheated to 60 °C. The cooling–heating process was repeated twice. The DSC thermogram of the suspension (30 μL) in a sealed aluminum pan was measured at a heating rate of 1 °C min^{-1} with the buffer as a reference on a computerized Shimadzu DSC-50 instrument. The heat and the temperature outputs were calibrated with 99.99% gallium (mp, 302.9 K; enthalpy of melting, 5.589 kJ/mol). The lipid and the peptide concentrations were 50 and 0–4.5 mM, respectively.

CD. Egg PC sonicated vesicles (0.5 mM) were prepared by use of twice-distilled water as a hydrating medium. Small aliquots (2.5% v/v) of a 2 mM hypelcin A/methanol solution were added to the vesicle suspension or water. The CD spectra were recorded on a Jasco 500 A instrument with a DP-501N data processor. The instrumental outputs were calibrated with nonhygroscopic ammonium *d*-camphor-10-sulfonate (Takakuwa et al., 1985). A quartz cuvette of 1- or 2-mm path length was thermostated at 30 ± 0.5 °C. Eight scans were averaged for each sample. Averaged blank spectra (the vesicle suspension or water) were subtracted to yield the "pure" spectra of the peptide. The reported spectra were the average of three independent preparations for each type of sample. The standard errors were indicated by error bars. The CD spectra of a poly(L-lysine) aqueous solution (46 μg/mL) were also measured in the absence or the presence of 0.5 mM egg PC/SA (9/1) sonicated vesicles to check effects of the light scattering due to the vesicles on the spectra.

Results

Leakage. Figure 1 shows the time courses of hypelcin A induced calcein leakage from the egg PC vesicles. In the absence of the peptide (only 3% v/v of methanol was added) no leakage was observed (open squares). Addition of the peptide caused the rapid release of the trapped dye. The leakage rate depended on both the peptide and the lipid con-

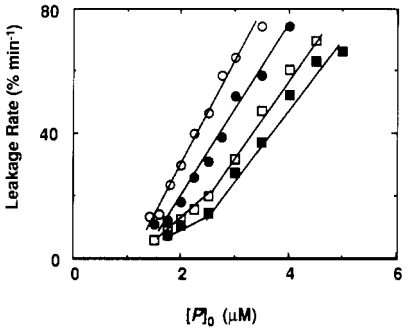

FIGURE 2: Dependence of calcein leakage rate on peptide and lipid concentrations for egg PC sonicated vesicles. The experiments were similar to those in Figure 1. The calcein leakage rate, defined as percent leakage for the initial first minute, is plotted as a function of $[P]_0$ at different [L]. [L] (μM): open circles, 107; closed circles, 160; open squares, 211; closed squares, 254.

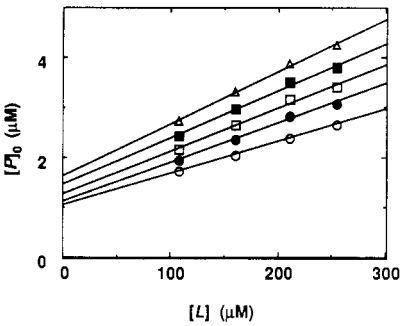

FIGURE 3: Estimation of free and membrane-bound peptide concentrations. Four pairs of $[P]_0$ and [L] values where a given leakage rate was observed were obtained from Figure 2. $[P]_0$ is plotted against [L] according to the equation in the text. The free peptide concentration, $[P]_f$, and the amount of the membrane-bound peptide per lipid molecule, r, were evaluated from the intercept and the slope, respectively. Leakage rate (% min^{-1}): open circles, 20; closed circles, 30; open squares, 40; closed squares, 50; triangles, 60. The lines are the least-squares fits.

centrations: an increase in the peptide concentration or a decrease in the lipid concentration resulted in an enhanced leakage rate, suggesting the involvement of a binding process in the leakage phenomenon. We examined this dependency more systematically (Figure 2) in order to estimate the free and the membrane-bound concentrations of the peptide (Matsuzaki et al., 1989). Here the leakage rate defined as percent leakage for the initial first minute is used as a measure of the initial leakage rate because the true rate was too rapid to measure precisely without a stopped-flow apparatus. We assumed that the leakage rate is determined only by the amount of the membrane-bound peptide per lipid molecule, r. The amount, r, can be connected to experimental conditions (the total peptide concentration, $[P]_0$, and the lipid concentration, [L]) through a material balance equation:

$$[P]_0 = [P]_f + r[L]$$

where $[P]_f$ is the free peptide concentration. A pair of r and $[P]_f$ values corresponding to a given leakage rate can be thus estimated with four sets of $[P]_0$ and [L] where the leakage rate was observed. Figure 3 depicts that the $[P]_0$ versus [L] plots according to the above equation at various rates indeed gave linear relations (the square correlation coefficients were greater than 0.990). The r and the $[P]_f$ values were obtained from the slopes and the intercepts, respectively. The relationship between r and $[P]_f$ thus estimated (i.e., binding isotherm) is shown in Figure 4a with the data for the DSPC vesicles. In Figure 4b the leakage rates are plotted as a function of r. There were several differences in the two vesicular systems. First, the binding of hypelcin A to the egg PC vesicles (Figure 4a, circles) was explained by partition (a linear binding isotherm with a slope of 6.6×10^3 M^{-1}). In contrast, for the DSPC vesicles, r is a higher order function of $[P]_f$ (squares). Second, the dye efflux from the DSPC vesicles (Figure 4b, squares) began at a much smaller r (7×10^{-4}) compared with that for the egg PC vesicles (circles, 5×10^{-3}). Third, the leakage rate increased linearly with r for the egg PC vesicles, whereas that for the DSPC vesicles was saturated.

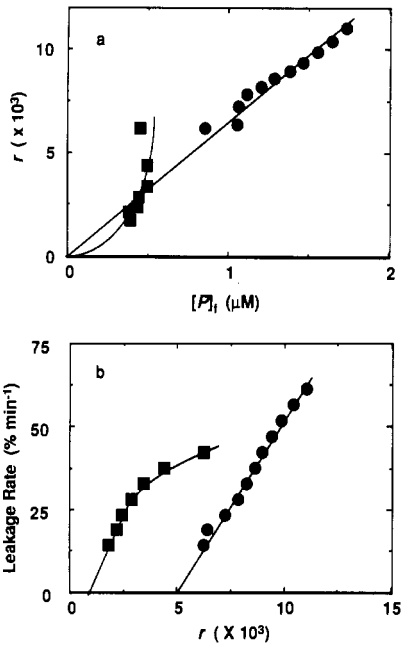

FIGURE 4: Quantitative analysis of hypelcin A induced leakage at 30 °C. The relationships (a) between r and $[P]_f$ (i.e., binding isotherm) and (b) between the leakage rate and r, obtained from Figure 3, are shown. Vesicles: circles, egg PC; squares, DSPC.

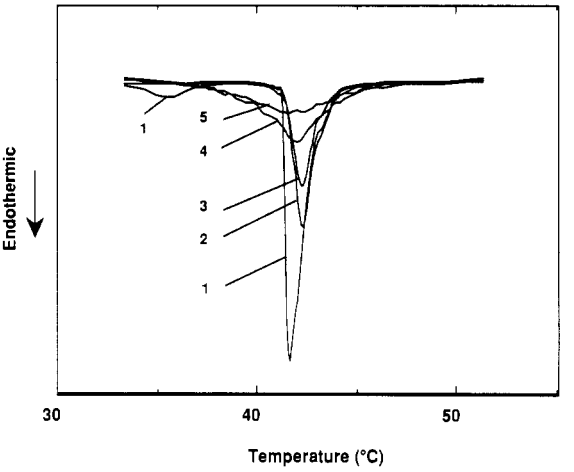

FIGURE 5: DSC heating curves of hypelcin A incorporated DPPC vesicles. The heating rate was 1 °C min^{-1}. Peptide content (mol %): curve 1, 0; curve 2, 1; curve 3, 2; curve 4, 5; curve 5, 7.

DSC. Effects of hypelcin A on the gel to liquid-crystalline phase transition were examined by the DSC technique. Figure 5 depicts the DSC heating curves of the DPPC vesicles at different peptide contents. In the absence of the peptide (curve 1), a sharp onset of the main transition appeared at 41.4 °C with a pretransition at 33.5 °C, typical of DPPC multilamellar vesicles (Wilkinson & Nagle, 1981). Incorporation of the peptide resulted in the disappearance of the pretransition, a reduced transition enthalpy, and a broadened DSC curve with a decreased transition-onset temperature (curves 2–5). The change in the transition temperature and the broadening be-

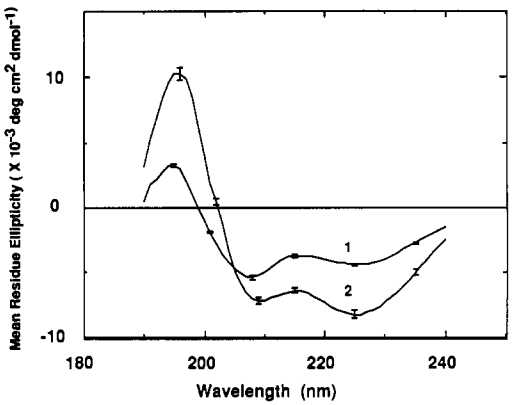

FIGURE 6: CD spectra of hypelcin A in the absence and the presence of egg PC sonicated vesicles. The CD spectra of a 25 μM hypelcin A aqueous solution were recorded in the absence (curve 1) and the presence (curve 2) of 0.5 mM egg PC sonicated vesicles at 30 °C. The error bars indicate standard errors for three independent preparations.

came significant around 5 mol % of hypelcin A.

CD. The conformational changes of hypelcin A upon binding to the lipid membrane were estimated on the basis of its CD spectra. Figure 6 shows that addition of egg PC vesicles appeared to change the CD spectrum drastically. Prior to interpretation of this spectral change, however, the contribution of optical artifacts should be clarified: In vesicle suspensions, CD spectra may be distorted by two optical effects, differential light scattering and differential absorption flattening (Gordon & Holzwarth, 1971; Mao & Wallace, 1984). The former can be minimized by use of small unilamellar vesicles (Mao & Wallace, 1984). The diameters of the egg PC vesicles used in the CD study were determined with the quasi-elastic light scattering instrument. The number-averaged diameters in the absence and the presence of hypelcin A were 25 and 37 nm, respectively, suggesting minimal scattering effects. Furthermore, we confirmed that the coexistence of the egg PC/SA vesicles (diameter, 41 nm) had no effects on the CD spectrum of the poly(L-lysine) aqueous solution (data not shown). Therefore, the CD spectrum of hypelcin A in the vesicle suspension was not distorted by the light scattering.

The other optical effect, absorption flattening, results from an inhomogeneous distribution of chromophores in a solution. The observed spectral change is in the direction opposite to the flattening. However, curve 2 per se might be somewhat distorted by this optical effect. The extent of flattening is related to the number of particles found in the light path (Gordon & Holzwarth, 1971). In the present experimental condition (0.5 mM vesicles of about 30 nm in diameter, 1-mm cuvette), the number (i.e., the *qm* value) is estimated at ca. 50, which is much larger than the absorbance of the corresponding solution (A_{sol}), indicating a negligible flattening effect. Reportedly, incorporation of proteins into small unilamellar vesicles at high lipid to protein ratios can minimize this optical artifact (Mao & Wallace, 1984). Thus, the spectral change upon addition of the vesicles is ascribable solely to the conformational change of the peptide.

The observed spectral change included conspicuousness of double minima around 209 and 225 nm and a maximum around 195 nm, suggesting an increase in the helical content. A similar change has been reported for alamethicin (Rizzo et al., 1987). The secondary structures of hypelcin A in the aqueous and lipidic media were estimated by a least-squares method (Yamaoka & Nakagawa, 1983) with the reference spectra of Chang et al. (1978). The only constraint employed was that each fraction (helix, β-sheet, turn, and unordered) should not be negative. The helical reference spectra depend on the helical length (n), which was varied (3.6, 5, 7.2, and 10). The best fits were obtained with an n value of 7.2. However, the other results were qualitatively the same. The calculated fractions were divided by the sum of the fractions for normalization. The fitting quality was evaluated with the normalized root mean standard deviation (NRMSD; Mao & Wallace, 1984). The presence of the lipid vesicles increased the helical content after normalization (33% or 42%) and reduced the unordered content (39% to 28%) with the fractions of the β-sheet (0% to 0%) and the turn (28% to 30%) unchanged. The sums and NRMSDs were 0.59 and 0.25 in the absence and 0.91 and 0.21 in the presence of the vesicles. The fitting was poor, and the sum for aqueous hypelcin A was rather small.

In the CD experiments the buffer components (Tris and NaCl), which may affect the conformation of hypelcin A in aqueous media, were omitted for measurement below 200 nm. The CD spectra of 5–50 μM hypelcin A buffer solutions were identical with curve 1 (Figure 6) in the wavelength range 200–240 nm (data not shown), indicating that the peptide is soluble in the buffer at least up to 50 μM with a fixed conformation which is unaffected by the presence of the buffer components.

Discussion

Hypelcin A shows antimicrobial and uncoupling activities (Fujita et al., 1984b, 1979; Takaishi et al., 1980), as do other Aib-containing peptides (Das et al., 1986; Mathew et al., 1982; Takaishi et al., 1980). These bioactivities may be explained in terms of the increased permeability of bacterial or mitochondrial membranes. Thus, the elucidation of hypelcin A–lipid vesicle interactions will serve to reveal the action mechanisms. Only a few reports have been published on the permeability change of lipid vesicles induced by alamethicin, a most extensively studied Aib-containing peptide, although a large body of experiments have been accumulated about the voltage-gated ion channel in black lipid membranes (Boheim, 1974; Eisenberg et al., 1973; Hall & Vodyanoy, 1984): Alamethicin and its synthetic fragments have divalent cation translocating effects in egg PC vesicles at a much higher concentration (10 μM; Mathew et al., 1982; Nagaraj et al., 1980) than that in the planer bilayer experiments (submicromolar; Eisenberg et al., 1973).

We have examined the effects of hypelcin A on the lipid bilayer permeability by using fluorescent dye loaded sonicated vesicles. Fluorescent dyes, such as calcein (Allen & Cleland, 1980) and 5/6-carboxyfluorescein (Weinstein et al., 1977), have been widely employed for investigating the permeability changes of the vesicles induced by peptides, proteins, or surfactants. However, recent studies (Bramhall, 1984; Bramhall et al., 1987) suggest that 5/6-carboxyfluorescein per se readily permeates the membranes and that the vesicles can retain the dye because of the creation of the transmembrane potential resulting from the much slower permeation rate of its counter ion (e.g., Na^+). Therefore, the increased dye permeability observed may imply enhanced *cation* permeability. To clarify this point in our experimental system, we studied the efflux of K^+ or calcein from the egg PC REVs induced by a cation-selective ionophore, gramicidin (Myers & Haydon, 1972). REVs having a larger trapped volume were employed because of the relatively low sensitivity of the K^+ electrode compared with that of the fluorescence method. Figure 7 shows that addition of gramicidin caused a rapid K^+ efflux (circles) but no calcein leakage (squares). This result clearly shows that the calcein leakage is not coupled by the counterion efflux and

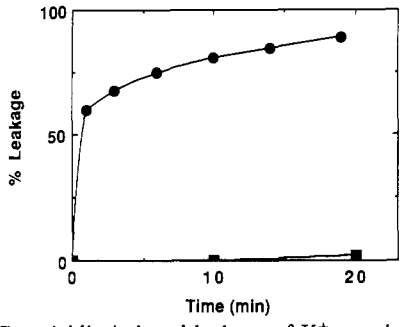

FIGURE 7: Gramicidin-induced leakage of K^+ or calcein from egg PC REVs. Small aliquots of gramicidin/methanol solution were added to egg PC REVs loaded with K^+ or calcein at 30 °C. The peptide and the lipid concentrations were 4 and 700 μM, respectively. The leakage of K^+ (circles) and calcein (squares) was followed with a K^+-selective electrode and a spectrofluorometer, respectively.

that the permeability of the dye per se is not as high as that of 5/6-carboxyfluorescein probably because calcein bears four negative charges in contrast to two charges of 5/6-carboxyfluorescein, at pH 7.0. Therefore, the hypelcin A induced calcein efflux implies the formation of a structural defect in the bilayers through which the relatively large dye molecule can move.

Knowledge on the intramembrane aggregation states of the peptide is important to elucidate the leakage mechanism. The binding isotherm will provide this information. Rizzo et al. (1987) revealed alamethicin aggregation in lipid vesicles on the basis of its binding isotherm obtained from CD measurements. We estimated the isotherm by using the indirect method based on the dependency of the leakage rate on the peptide and the lipid concentrations (Matsuzaki et al., 1989). This method has been employed for studying hemolysis (Matsuzaki et al., 1988; Thron, 1964) and surfactant–vesicle interactions (Paternostre et al., 1988). The only reasonable assumption is that the amount of the membrane-bound peptide per lipid molecules, r, determines the leakage rate. The linearity in Figure 3 demonstrates that an r value specifies the corresponding $[P]_f$ value, suggesting the establishment of the binding equilibrium. Otherwise, there is not experimental condition independent relationship between r and $[P]_f$ at a given time (in this case, 1 min).[2] Therefore, the leakage kinetics (Figure 1) represents that of a postbinding process. Indeed, recent stopped-flow experiments clarified that the binding of small peptides, such as alamethicin (Schwartz et al., 1987) or melittin (Schwarz & Beschiaschvili, 1989), to the lipid vesicles is a very fast process (millisecond order).

The linear isotherm for the egg PC vesicles (Figure 4a, circles) indicates that the intramembrane peptide molecules are present mainly as the monomer if the monomeric peptide is the only species in the aqueous phase. This assumption is compatible with the concentration-independent CD spectra of the peptide in the buffer. The slope of the isotherm, i.e., the partition coefficient (6.6×10^3 M^{-1}) in comparable with the value estimated for alamethicin (Rizzo et al., 1987). On the other hand, hypelcin A molecules aggregate in the solid DSPC vesicles[3] because the isotherm has an increasing slope (Figure 4a, squares). The leakage rate–r relationship for the DSPC vesicles (Figure 4b, squares) shows that the rate is saturated as the peptide molecules aggregate. These findings indicate that the *monomeric* peptide causes the increased permeability. The leakage-onset r value for the DSPC vesicles (7×10^{-4}) corresponds to ca. three molecules per vesicle, which is too small to form a monomer-aggregated pore for the dye to pass through. This additional fact excludes the pore mechanism as proposed for the alamethicin ion channel (Fox & Richards, 1982). The membrane-perturbing activity of hypelcin A is much higher compared with that of magainin 1, an antibiotic peptide, for bovine brain phosphatidylserine-sonicated vesicles (Matsuzaki et al., 1989; the leakage occurs at $r \approx 0.03$) or that of surfactants for egg PC REVs (Triton X-100, $r \approx 0.05$; sodium cholate, $r \approx 0.1$; Paternostre et al., 1988).

The monomer mechanism previously discussed will involve a peptide-induced disruption of the lipid packing in the bilayers. The modification of the lipid–lipid interactions affects the phase transition behavior of the membranes. Our DSC results indicate that the incorporation of hypelcin A into the DPPC bilayers leads to a reduced enthalpy change and a broadened transition. These phenomena, often observed for peptides or proteins interacting with the hydrophobic region of the membranes, have been interpreted in terms of "boundary lipids" (Alonso et al., 1982; Chapman et al., 1977; Gomez-Fernandez et al., 1980; Semin et al., 1984), although a "two-phase coexistence model" may also explain the results (Morrow et al., 1985). We found, by a polarized Fourier transform infrared-attenuated total reflection spectroscopy technique, that the presence of 5 mol % hypelcin A solidifies DPPC molecules above its transition temperature in a conformation similar to that in the gel phase (Matsuzaki et al., unpublished work). Thus, the peptide incorporation may result in a lipid packing perturbation including a "phase separation". The details are under investigation. The transition enthalpy versus peptide content plot may be used to estimate the amount of boundary lipids (Alonso et al., 1982; Chapman et al., 1977; Gomez-Fernandez et al., 1980; Semin et al., 1984), but we did not adopt this approach because such a simple treatment may lead to a misinterpretation without sufficient data on the thermodynamics of the peptide–lipid system (Morrow et al., 1985).

Information on the peptide conformation in the membranes is essential to molecular-level understanding of the peptide–lipid interactions. Our CD results show that the conformation of hypelcin A changes drastically upon membrane binding. Analysis of the two CD spectra in Figure 6 provides some information of this conformational change, although curve 2 includes some contribution from free peptide (i.e., curve 1). The extent of the contribution cannot be evaluated because the experimental condition for the CD study was beyond the binding isotherm (Figure 4a), even if the absence of the buffer components did not affect the isotherm. We estimated the peptide conformations with and without the vesicles by using the least-squares method. Addition of the vesicles increases the helical content at the expense of the reduced unordered content. However, the quantitative estimate for the enhanced

[2] For example, we consider the partition process of the peptide to the membrane. The entrance and the exit rate constants are denoted by k_1 and k_{-1}, respectively. Solving the differential kinetic equations with an initial condition of $r = 0$ at $t = 0$ (t, time) gives

$$\frac{r}{[P]_f} = \nu_L \frac{1 - \exp\{-(k_{-1} + \nu_L[L]k_1)t\}}{k_{-1}/k_1 + \nu_L \exp\{-(k_{-1} + \nu_L[L]k_1)t\}}$$

where ν_L is the partial molar volume of the membrane phase. The $r/[P]_f$ value depends on an experimental condition, [L], at any finite time.

[3] Sonication of multilamellar vesicles is known to reduce the phase transition temperature and to broaden the transition. Sonicated DPPC vesicles undergo the transition in the temperature range 29.7–40.6 °C (Lentz et al., 1976). Thus, in our experimental condition (30 °C), the DPPC sonicated vesicles are around the onset of the transition, whereas the DSPC sonicated vesicles employed here are in the gel state.

folding was small in spite of the large spectral change. This is ascribable to the small sum of the fractions for curve 1. The small sum may be caused by the possibility that small peptides adopt multiple conformations that result in net ellipticities near zero, instead of defined periodic structures, owing to their flexibility, especially in solution states (Cascio & Wallace, 1988). That the small sum resulted from the peptide loss due to adsorption can be denied because of the concentration independency of the spectra. Furthermore, the fitting was unsatisfactory. Similar problems have been reported for alamethicin (Cascio & Wallace, 1988; Vogel, 1987). The poor results may arise, in addition to the peptide flexibility, from the existence of some structures different from the four reference structures, such as 3_{10} or a distorted helix (Cascio & Wallace, 1988; Vogel, 1987), and/or by spectral shifts due to the medium polarity effect (Cascio & Wallace, 1988). In spite of the problems mentioned above, we can conclude that the binding of hypelcin A to the lipid membranes accompanies a conformational change including an increase in the helical content. Membrane-associating peptides tend to fold in helical conformations in the lipid environments (Drake & Hider, 1979; Kaiser & Kézdy, 1984; Kanellis et al., 1980; Matsuzaki et al., 1989).

In summary, hypelcin A binds to the lipid vesicles with enhanced helical folding. The bound monomeric peptide strongly interacts with the surrounding lipids to cause a packing defect, leading to the increased permeability. This defect may be similar to that formed in a phase transition temperature region where the membranes are permeable to relatively large molecules such as poly(ethylene glycol) 900 (Van Hoogevest et al., 1984). This scheme for the leakage well explains the ineffective membrane perturbation of the aggregated peptide: the lipid–peptide contact area per peptide molecule decreases with the aggregation. The membrane-perturbing activity of the monomeric peptide is higher for the DSPC vesicles, indicating the packing defect cannot be repaired by the rigid DSPC molecules as effectively as by the flexible egg PC molecules. The fusion of the vesicles, as observed for alamethicin (Lau & Chan, 1974), is a plausible leakage mechanism. Indeed, hypelcin A induces the fusion, but the r value required to cause the fusion is slightly larger than that for the leakage (Matsuzaki et al., unpublished work), excluding the fusion as the leakage mechanism. We will further investigate hypelcin A–lipid interactions to reveal its complete profile.

Registry No. PC, 28319-77-9; DPPC, 63-89-8; DSPC, 816-94-4; hypelcin A, 69910-29-8.

References

Allen, T. M., & Cleland, L. G. (1980) *Biochim. Biophys. Acta 597*, 418–426.

Alonso, A., Restall, C. J., Turner, M., Gomez-Fernandez, J. C., Goñi, F. M., & Chapman, D. (1982) *Biochim. Biophys. Acta 689*, 283–289.

Banerjee, U., Zidovetzki, R., Birge, R. R., & Chan, S. I. (1985) *Biochemistry 24*, 7621–7627.

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

Boheim, G. (1974) *J. Membr. Biol. 19*, 277–303.

Boheim, G., Janko, K., Leibfritz, D., Ooka, T., König, W. A., & Jung, G. (1976) *Biochim. Biophys. Acta 433*, 182–199.

Bramhall, J. (1984) *Biochim. Biophys. Acta 778*, 393–399.

Bramhall, J., Hofmann, J., DeGuzman, R., Montestruque, S., & Schell, R. (1987) *Biochemistry 26*, 6330–6340.

Cascio, M., & Wallace, B. A. (1988) *Proteins: Struct., Funct., Genet. 4*, 89–98.

Chang, C. T., Wu, C.-S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13–31.

Chapman, D., Cornell, B. A., Eliasz, A. W., & Perry, A. (1977) *J. Mol. Biol. 113*, 517–538.

Das, M. K., Raghothama, S., & Balaram, P. (1986) *Biochemistry 25*, 7110–7117.

Drake, A. F., & Hider, R. C. (1979) *Biochim. Biophys. Acta 555*, 371–373.

Eisenberg, M., Hall, J. E., & Mead, C. A. (1973) *J. Membr. Biol. 14*, 143–176.

Epand, R. M., Epand, R. F., Orlowski, R. C., Schlueter, R. J., Boni, L. T., & Hui, S. W. (1983) *Biochemistry 22*, 5074–5084.

Fox, R. O., Jr., & Richards, F. M. (1982) *Nature 300*, 325–330.

Fujita, T., Takaishi, Y., & Shiromoto, T. (1979) *J. Chem. Soc., Chem. Commun.*, 413–414.

Fujita, T., Takaishi, Y., Matsuura, K., Takeda, Y., Yoshioka, Y., & Brückner, H. (1984a) *Chem. Pharm. Bull. 32*, 2870–2873.

Fujita, T., Takaishi, Y., Moritoki, H., Ogawa, T., & Tokimoto, K. (1984b) *Chem. Pharm. Bull. 32*, 1822–1828.

Gomez-Fernandez, J. C., Goni, F. M., Bach, D., Restall, C. J., & Chapman, D. (1980) *Biochim. Biophys. Acta 598*, 502–516.

Gordon, D. J., & Holzwarth, G. (1971) *Arch. Biochem. Biophys. 142*, 481–488.

Hall, J. E., & Vodyanoy, I. (1984) *Biophys. J. 45*, 233–247.

Irmscher, G., & Jung, G. (1977) *Eur. J. Biochem. 80*, 165–174.

Kaiser, E. T., & Kézdy, F. J. (1984) *Science 223*, 249–255.

Kanellis, P., Romans, A. Y., Johnson, B. J., Kercret, H., & Chiovetti, R., Jr. (1980) *J. Biol. Chem. 255*, 11464–11472.

Kubesch, P., Boggs, J., Luciano, L., Maass, G., & Tümmler, B. (1987) *Biochemistry 26*, 2139–2149.

Lau, A. L. Y., & Chan, S. I. (1974) *Biochemistry 13*, 4942–4948.

Lentz, B. R., Barenholz, Y., & Thompson, T. E. (1976) *Biochemistry 15*, 4521–4528.

Mao, D., & Wallace, B. A. (1984) *Biochemistry 23*, 2667–2673.

Mathew, M. K., Nagaraj, R., & Balaram, P. (1982) *J. Biol. Chem. 257*, 2170–2176.

Matsuzaki, K., Handa, T., Miyajima, K., Mikura, Y., Shimizu, H., & Toguchi, H. (1988) *Chem. Pharm. Bull. 36*, 4253–4260.

Matsuzaki, K., Harada, M., Handa, T., Funakoshi, S., Fujii, N., Yajima, H., & Miyajima, K. (1989) *Biochim. Biophys. Acta 981*, 130–134.

Morrow, M. R., Huschilt, J. C., & Davis, J. H. (1985) *Biochemistry 24*, 5396–5406.

Myers, V. B., & Haydon, D. A. (1972) *Biochim. Biophys. Acta 274*, 313–322.

Nagaraj, R., Mathew, M. K., & Balaram, P. (1980) *FEBS Lett. 121*, 365–368.

Paternostre, M.-T., Roux, M., & Rigaud, J.-L. (1988) *Biochemistry 27*, 2668–2677.

Rizzo, V., Stankowski, S., & Schwartz, G. (1987) *Biochemistry 26*, 2751–2759.

Schwarz, G., & Beschiaschvili, G. (1989) *Biochim. Biophys. Acta 979*, 82–90.

Schwartz, G., Gerke, H., Rizzo, V., & Stankowski, S. (1987) *Biophys. J. 52*, 685-692.
Semin, B. K., Saraste, M., & Wikström, M. (1984) *Biochim. Biophys. Acta 769*, 15-22.
Subbarao, N. K., Parente, R. A., Szoka, F. C., Jr., Nadasdi, L., & Pongracz, K. (1987) *Biochemistry 26*, 2964-2972.
Szoka, F., Jr., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194-4198.
Takaishi, Y., Terada, H., & Fujita, T. (1980) *Experientia 36*, 550-551.
Takakuwa, T., Konno, T., & Meguro, H. (1985) *Anal. Sci. 1*, 215-218.
Thron, C. D. (1964) *J. Pharm. Exp. Ther. 145*, 194-201.
Van Hoogevest, P., de Gier, J., & de Kruijff, B. (1984) *FEBS Lett. 171*, 160-164.
Vogel, H. (1987) *Biochemistry 26*, 4562-4572.
Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R., & Hagins, W. A. (1977) *Science 195*, 489-492.
Wilkinson, D. A., & Nagle, J. F. (1981) in *Liposomes* (Knight, C. G., Ed.) pp 273-298, Elsevier/North-Holland Biomedical Press, Amsterdam.
Yamaoka, K., & Nakagawa, T. (1983) *J. Pharm. Dyn. 6*, 595-606.

Dependence of the Bilayer to Hexagonal Phase Transition on Amphiphile Chain Length†

Richard M. Epand,* Kelli S. Robinson, Marie E. Andrews, and Raquel F. Epand
Department of Biochemistry, McMaster University Health Sciences Centre, Hamilton, Ontario L8N 3Z5, Canada
Received January 19, 1989; Revised Manuscript Received July 28, 1989

ABSTRACT: Several series of amphiphiles of increasing chain length were tested for their abilities to modify the L_α-H_{II} transition of dielaidoylphosphatidylethanolamine using differential scanning calorimetry. Acylcarnitines, alkyl sulfates, alkylsulfobetaines, and phosphatidylcholines, with chain lengths between about 6 and 12 carbon atoms, show an increasing capacity to raise the L_α-H_{II} phase transition temperature of phosphatidylethanolamine. This is ascribed to increased partitioning of the added amphiphile from water into the membrane as the chain length increases. Alkyl sulfates and alkyltrimethylammonium bromides have diminished capacities to raise the L_α-H_{II} transition temperature as the chain length is increased from 12 to 16. This is caused by an increase in the hydrophobic portion of the amphiphile leading to a change in the intrinsic radius of curvature and a decrease in the hydrocarbon packing constraints in the H_{II} phase relative to the shorter chain amphiphiles. The L_α-H_{II} transition temperature of phosphatidylethanolamine with acylcarnitines of chain length 14-20 carbon atoms, alkylsulfobetaines above 14 carbon atoms, and phosphatidylcholines with acyl groups having above 10 carbon atoms is relatively insensitive to chain length. We suggest that this is caused by a balance between increasing hydrocarbon volume promoting the H_{II} phase through decreased intrinsic radius of curvature and greater relief of hydrocarbon packing constraints vs greater intermolecular interactions favoring the more condensed L_α phase. This latter effect is more important for amphiphiles with large headgroups which can pack more efficiently in the L_α phase. The phosphatidylcholines show a gradual decrease in bilayer stabilization between 10 and 22 carbon atoms. This effect is greater in the two-chain amphiphiles than in the single-chain amphiphiles which have only half as much hydrocarbon for the same chain length. Acylcarnitines above a chain length of 20 exhibit a decrease in bilayer stabilization.

The phospholipids of biological membranes are normally organized as bilayers (L_α phase). However, it has long been recognized that some lipid components of these membranes spontaneously form an inverted hexagonal phase structure (H_{II} phase) composed of cylindrical micelles with an aqueous core (Cullis & De Kruijff, 1978; Dekker et al., 1983). It is still controversial as to whether structures resembling the H_{II} phase are intermediates in membrane function. However, it is clear that membrane function can be modulated by substances which affect L_α-H_{II} interconversions. For example, membrane fusion is inhibited by substances which favor the L_α phase. A bilayer stabilizer may favor the L_α phase either by lowering the energy of the L_α phase or by raising the energy of the H_{II} phase. A number of these bilayer-stabilizing compounds have been shown to have antiviral activity including carbobenzoxy-D-Phe-L-Phe-Gly (Richardson et al., 1980; Epand, 1986), cyclosporin A (Gui et al., 1983; McKenzie et al., 1987), tromantadine (Lieb & May, 1972; Cheetham & Epand, 1987), carbobenzoxy-L-Ser-L-Leu-amide (Epand et al., 1987), carbobenzoxy-D-Phe-L-Leu-Gly-D-Phe-D-Leu-D-Leu, and carbobenzoxy-D-Phe-L-Leu-Gly-D-Phe-D-Leu-D-Leu-Gly (Lobl et al., 1988). In addition, the activity of several membrane-bound enzymes is altered by substances which affect L_α-H_{II} interconversion. For example, protein kinase C is inhibited by uncharged and zwitterionic compounds which are bilayer stabilizers (Epand, 1987; Epand et al., 1988, 1989).

To further understand the factors which affect the L_α-H_{II} transition, we have undertaken a systematic study of several series of amphiphiles of varying chain length. The L_α-H_{II} transition temperature of phosphatidylethanolamines is particularly susceptible to the presence of low concentrations of certain amphiphiles (Epand, 1985). The series of amphiphiles we use in the present study are alkyltrimethylammonium

† This work was supported by a grant from the Medical Research Council of Canada.

0006-2960/89/0428-9398$01.50/0