On the Mechanism of Inhibition of Viral and Vesicle Membrane Fusion by Carbobenzoxy-D-phenylalanyl-L-phenylalanylglycine†

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ABSTRACT: The mechanism by which the hydrophobic peptide Z-D-Phe-L-PheGly inhibits membrane fusion was investigated. Differential scanning calorimetry, ^2H nuclear magnetic resonance (NMR), and ^{13}C NMR of phosphatidylcholine bilayers in the presence of Z-D-Phe-L-PheGly indicate that this hydrophobic peptide penetrates the phospholipid bilayer but does not strongly perturb the properties of phosphatidylcholine bilayers with a large effective radius of curvature. However, Z-D-Phe-L-PheGly does affect the properties of highly curved phosphatidylcholine bilayers. Small, sonicated vesicles (SUV) of dipalmitoylphosphatidylcholine (DPPC) were made in the presence and absence of Z-D-Phe-L-PheGly. At pH 4.5, the presence of Z-D-Phe-L-PheGly inhibited the formation of SUV. At pH 6.7 and 7.4, SUV could form. Once sonication ceased and the vesicles were incubated at 23 °C, modest growth in vesicle size occurred for pure DPPC SUV. Rapid change to large sheetlike structures occurred in the presence of Z-D-Phe-L-PheGly. Z-D-Phe-L-PheGly appeared to favor the formation of phospholipid structures with large radii of curvature. In these experiments, Z-D-Phe-L-PheGly had access to both sides of the bilayer. If Z-D-Phe-L-PheGly was added to preformed DPPC SUV (and thus present initially only in the outer leaflet of the vesicle bilayer) and incubated at 23 °C, only modest growth in vesicle size was observed with little difference from control values at short to intermediate incubation times. From these data, it was suggested that Z-D-Phe-L-PheGly stabilized phospholipids in structures with large radii of curvature and in particular destabilized assemblies of phospholipids with a "negative" radius of curvature. The most likely method of inhibition of membrane fusion by Z-D-Phe-L-PheGly was the inhibition of the formation of intermediate(s) along the pathway of membrane fusion that involved phospholipid structures with small, "negative" radii of curvature.

Small hydrophobic peptides were reported to inhibit infection by several enveloped viruses, including measles and herpes simplex virus (Nicolaides et al., 1968; Richardson & Choppin, 1983; Richardson et al., 1980). The prototypical inhibitory peptide of this class, carbobenzoxy-D-phenylalanyl-L-phenylalanylglycine (Z-D-Phe-L-PheGly),[1] was among the most potent at inhibiting infection. This class of compounds has been observed to inhibit membrane fusion between large unilamellar vesicles (LUV) of *N*-methyldioleoylphosphatidylethanolamine (*N*-methyl-DOPE), between Sendai virus and *N*-methyl-DOPE LUV, and between Sendai virus and resealed erythrocyte ghosts (Kelsey et al., 1990, 1991). The relative potency of these peptides at inhibiting membrane fusion events was the same as the relative potency of the same group of peptides at inhibiting viral infection. Z-D-Phe-L-PheGly was among the most potent at inhibiting membrane fusion. Therefore, it was suggested that the inhibition of infection by these peptides was due to the inhibition of the fusion event between enveloped virus and cell membrane required as part of the pathway of entry of enveloped viruses into the target cell.

The purpose of this study was to better understand the mechanism by which peptides such as Z-D-Phe-L-PheGly inhibit membrane fusion. Previous ^{31}P nuclear magnetic resonance (NMR) studies suggested that the target of the inhibitory peptides was a putative intermediate in membrane fusion characterized by isotropic ^{31}P NMR resonances (Kelsey et al., 1991). These studies suggested that the effect of the peptide was to increase the radius of curvature of the highly curved structures that gave rise to the isotropic ^{31}P NMR resonances. Since structures with a small radius of curvature have been hypothesized to be on the pathway of membrane fusion (Siegel, 1987; Siegel et al., 1989), this effect of Z-D-Phe-L-PheGly on phospholipid packing and membrane structure suggested itself as a possible mechanism of inhibition of membrane fusion.

In this study, the effects of Z-D-Phe-L-PheGly on the packing of phospholipids in bilayers were investigated. Differential scanning calorimetry studies and ^2H NMR and ^{13}C NMR studies together revealed little effect of Z-D-Phe-L-PheGly on the structure of bilayers with a large radius of curvature. However, strong effects of Z-D-Phe-L-PheGly on bilayers with a small radius of curvature were noted. In particular, Z-D-Phe-L-PheGly appeared to preferentially destabilize phospholipid assemblies with a "negative" radius of curvature.

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[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; LUV, large unilamellar vesicle(s); *N*-methyl-DOPE, *N*-methyldioleoylphosphatidylethanolamine; NMR, nuclear magnetic resonance; ^{31}P NMR, ^{31}P nuclear magnetic resonance; PC, phosphatidylcholine; SUV, small unilamellar vesicle(s); Z-D-Phe-L-PheGly, carbobenzoxy-D-phenylalanyl-L-phenylalanylglycine.

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Materials and Methods

N-Methyldioleoylphosphatidylethanolamine (*N*-methyl-DOPE) and dipalmitoylphosphatidylcholine (DPPC) were obtained from Avanti Polar Lipids, Birmingham, AL. Dioleoylphosphatidylcholine (^{13}C-enriched in the *sn*-2 carbonyl) was synthesized by Avanti Polar Lipids. [9,10-^{2}H]Dioleoylphosphatidylcholine was obtained from Cambridge Isotopes. Carbobenzoxy-D-Phe-L-PheGly (Z-D-Phe-L-PheGly) and carbobenzoxy-Gly-L-Phe were purchased from Sigma (St. Louis, MO).

Phosphate Assay. Phosphate was determined by published procedures (Bartlett, 1959).

Sonication. Sonication was performed with a Branson W350 probe sonicator with an ice bath. First, multilamellar liposomes containing the peptide in the phospholipid bilayer were prepared in the following manner. In some experiments, dipalmitoylphosphatidylcholine (DPPC) and Z-D-Phe-L-PheGly were cosolubilized in chloroform/methanol (2:1) at room temperature in the indicated mole ratios. The solvent was removed by evaporation under a stream of nitrogen gas followed by evaporation under high vacuum overnight. The material was then hydrated in D_2O with 50 mM NaCl, sealed under nitrogen gas, and vortexed vigorously. The membranes were then sonicated with 5-min sonications followed by a 1-min rest period in which the light scattering of the sample was determined as the effective absorbance at 350 nm. In other experiments, DPPC SUV were formed without Z-D-Phe-L-PheGly, and the peptide was added subsequently in a small amount of methanol. All sonication experiments were performed a minimum of 2 or 3 times with the same results.

Differential Scanning Calorimetry. Dipalmitoylphosphatidylcholine (DPPC) and varying quantities of Z-D-Phe-L-PheGly peptide were dissolved together in chloroform/methanol (2:1 v/v). The solvent was evaporated with a stream of nitrogen so as to deposit the solutes as a film on the wall of a glass test tube. Final traces of solvent were removed in a vacuum desiccator at 30 °C for 2 h. Buffer at pH 7.4, composed of 20 mM Pipes, 150 mM NaCl, and 1 mM EDTA with 0.002% NaN_3, was added to each lipid film, and the tube was vortexed to form a suspension. The final concentration of lipid was 5 mg/mL, with mole fractions of peptide varying from 0 to 0.23.

Lipid suspensions were degassed under vacuum before being loaded into an MC-2 high-sensitivity differential scanning calorimeter (Microcal, Amherst, MA). A heating scan rate of 43 °C/h was used. The pretransition and the main transition were fitted to a single van't Hoff component, and the transition temperature was reported as that for the fitted curve.

Freeze–Fracture Electron Microscopy. For the results presented in Figure 7, samples were taken as a function of time from a preparation that was freshly sonicated at 50 °C. The sample for time zero was directly frozen from elevated temperature as quickly as possible after termination of the sonication. Subsequent samples were frozen from 23 °C, the temperature of incubation for the "growth" experiments. A 0.1-μL sample was placed in a thin copper sandwich holder, and was rapidly immersed in liquid propane using a plunger/damper device as described previously (Hui et al., 1981a). Fracturing was carried out at −120 °C in a Polaron unit equipped with Cressington E-beam guns. The replicas were examined in a Hitachi H-600 electron microscope.

NMR Measurements. ^{2}H NMR (41.4 MHz) and ^{13}C NMR (67 MHz) data were obtained on a JEOL FX270 multinuclear Fourier transform NMR spectrometer in 10-mm tubes, using a 50-kHz window. A total of 2048 data points were collected in the time domain. For ^{2}H NMR spectra, data were collected with the quadrupole echo sequence and repeat times of 100 ms. ^{13}C NMR spectra were obtained with a Hahn echo and a 1-s repeat time.

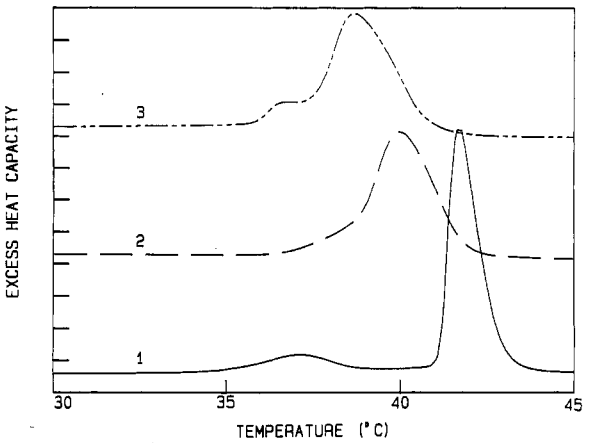

FIGURE 1: DSC heating scans of DPPC containing a mole fraction of Z-D-Phe-L-PheGly of 0 (curve 1), 0.055 (curve 2), or 0.238 (curve 3). Lipid concentration is 7 mM, and scan rate is 0.7 K/min. Curves are displaced from one another for convenience of display. Each division on the ordinate corresponds to an excess heat capacity of 1 kcal K^{-1} (mol of DPPC)$^{-1}$.

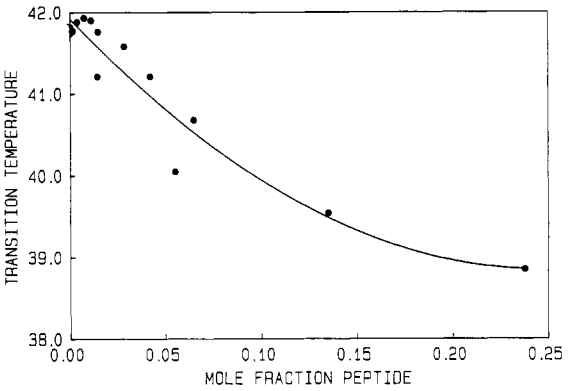

FIGURE 2: Temperature of maximum excess heat capacity as a function of the mole fraction of Z-D-Phe-L-PheGly admixed with DPPC.

Results

Effects of Z-D-Phe-L-PheGly on Phospholipid Bilayers with Large Radii of Curvature. Addition of Z-D-Phe-L-PheGly to DPPC caused a slight lowering of the $P_{\beta'}$–L_{α}-phase transition temperature. Figure 1 shows the relationship between this transition temperature and the mole fraction of Z-D-Phe-L-PheGly in the DPPC multilamellar liposomes. Even at a mole fraction of peptide of 0.25, the shift of the temperature of maximum heat capacity was only about 3 °C. Under the conditions of these experiments, Z-D-Phe-L-PheGly has been found to reside essentially entirely in the lipid bilayer. This was determined using partition experiments described previously (Kelsey et al., 1991), with the modification that the peptide was added to the lipid prior to hydration into bilayers.

Figure 2 shows representative DSC scans of DPPC multilamellar liposomes containing Z-D-Phe-L-PheGly. At low mole fractions, the premelt transition is considerably broadened, even at mole fractions as low as 0.014, indicating that the peptide is largely partitioning into the lipid. At a peptide mole fraction of 0.055, the premelt transition of DPPC is not detectable. This loss of the premelt transition is common to the addition of a number of lipophilic compounds to DPPC bilayers. At higher mole fractions of peptide, the transition shape becomes more complex, with a low-temperature shoulder. The total

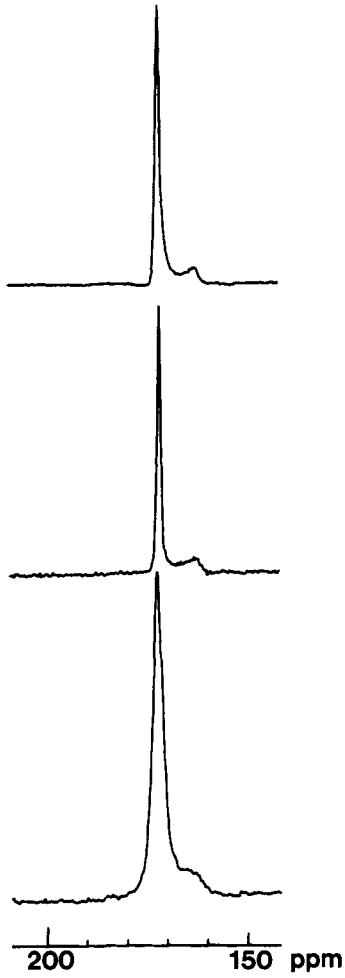

FIGURE 3: 67-MHz ^{13}C NMR spectra of 50 mg of dioleoylphosphatidylcholine (^{13}C-enriched in the *sn*-2 carbonyl) in multilamellar liposomes in 10 mM phosphate, pH 7. Spectra were obtained with a 50-kHz spectral width. The smallest division on the scale is 10 ppm. The top panel is pure phospholipid. The middle panel is phospholipid/Z-D-Phe-L-PheGly (8:1 mole ratio). The bottom panel is phospholipid/Z-D-Phe-L-PheGly (3:1 mole ratio).

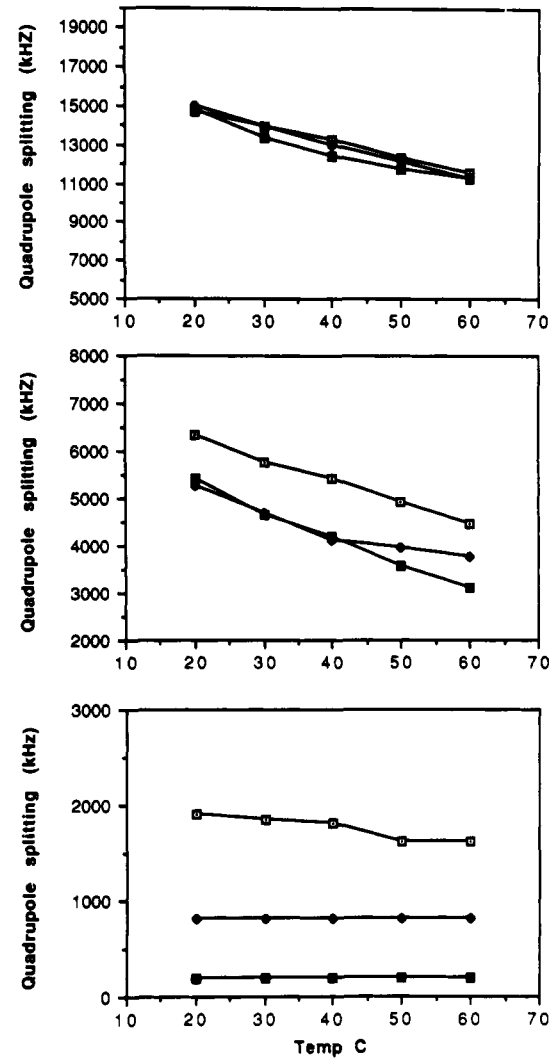

FIGURE 4: Effect of Z-D-Phe-L-PheGly on [9,10-^2H]DOPC. ^2H quadrupole splittings for [9,10-^2H]dioleoylphosphatidylcholine in multilamellar liposomes in 5 mM HEPES/50 mM NaCl, pH 7, with 16 mg of lipid in each sample, as a function of temperature. The open boxes represent the pure phospholipid, the closed diamonds represent phospholipid/Z-D-Phe-L-PheGly (8:1 mole ratio), and the closed boxes represent phospholipid/Z-D-Phe-L-PheGly (3:1 mole ratio). The top panel shows the quadrupole splittings from the deuterium at position 9. The middle panel shows the quadrupole splittings from the deuterium at position 10 on the *sn*-1 chain, and the bottom panel shows the quadrupole splittings from the deuterium at position 10 on the *sn*-2 chain.

enthalpy of the transition (average = 8.3 kcal/mol) remains constant even up to these high mole fractions of peptide, and the width of the transition is increased only about 2-fold. The van't Hoff enthalpies, by curve fitting using Microcal software, are 750, 341, and 340 kcal/mol for mole fractions of peptide of 0, 0.055, and 0.238, respectively. While these changes in the calorimetric parameters induced by Z-D-Phe-L-PheGly are experimentally significant, these results indicate that the peptide does not greatly perturb the packing arrangement of DPPC in multilamellar liposomes when it partitions into these phospholipid bilayers.

The effects of Z-D-Phe-L-PheGly on phospholipid properties were also investigated using NMR. ^{13}C NMR of dioleoylphosphatidylcholine, enriched in ^{13}C in the *sn*-2 carbonyl, was dispersed as multilamellar liposomes containing Z-D-Phe-L-PheGly (added with the phospholipid in organic solvent prior to hydration). The ^{13}C powder pattern from this carbonyl is normally collapsed due to a fortuitous orientation of this particular carbonyl relative to the director of the bilayer (Lewis et al., 1984). The half-height line width is about 1.2 ppm in these spectra. The line shape of the resonance from this carbonyl should be very sensitive to any change in conformation of the *sn*-2 chain; any alteration from the magic angle for this carbonyl would cause an expression of the ^{13}C chemical shift tensor and thus a broadening of the observed resonance. Figure 3 shows little if any effect on the ^{13}C resonance line width of the presence of Z-D-Phe-L-PheGly (8:1 phospholipid/peptide, 1.1 ppm half-height line width) but a broadening of the resonance at 3:1 (phospholipid/peptide, 3 ppm half-height line width) due to the expression of the ^{13}C chemical shift tensor of this carbonyl in the line width. Thus, there may be an alteration of the conformation of the carbonyl in the interfacial region due to the presence of the inhibitory peptide.

^2H NMR of [9,10-^2H]dioleoylphosphatidylcholine was then examined for possible effects of Z-D-Phe-L-PheGly on the hydrocarbon chains. The spectra of this specifically deuterated phospholipid consist of three overlapping quadrupole splittings as described elsewhere (Seelig et al., 1981). Figure 4 shows the numerical values of these quadrupole splittings as a function of temperature and as a function of the concentration of Z-D-Phe-L-PheGly in the bilayer. The largest quadrupole splittings, graphed in the top panel of Figure 4, are dominated by the molecular order parameter (Seelig et al., 1981) and

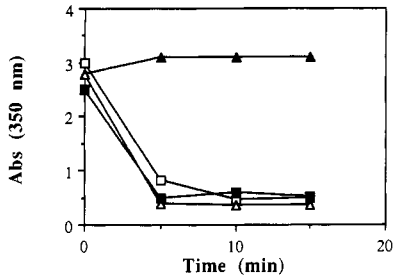

FIGURE 5: Sonication of 25 mg of DPPC/Z-D-Phe-L-PheGly (3:1 mole ratio) as a function of time in 50 mM NaCl/5 mM HEPES and as a function of pH. (▲) pH 4.5; (■) pH 6.7; (△) pH 7.4; (□) no peptide.

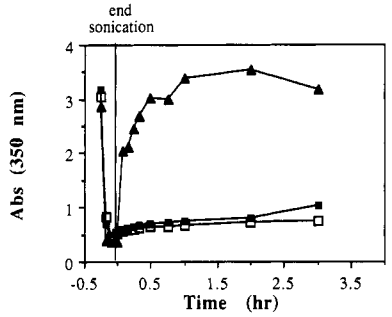

FIGURE 6: "Growth" of DPPC SUV (25 mg of phospholipid) after sonication as described in the text [experiment of Schullery et al. (1980)] with and without peptides. The peptides were mixed with DPPC in organic solvent prior to the formation of the vesicles by sonication. SUV were formed at 50 °C and were subsequently incubated at 23 °C. (□) Pure DPPC; (▲) DPPC/Z-D-Phe-L-PheGly (4:1 mole ratio); (■) DPPC/Z-GlyPhe (4:1 mole ratio).

show little influence of the peptide. The bottom panel in Figure 4 shows the smallest quadrupole splitting which is dominated by the orientation of the C–D bond. This quadrupole splitting is significantly affected by the presence of Z-D-Phe-L-PheGly. The middle panel shows mixed results from a quadrupole splitting which has significant contributions from both motional order and orientation of the C–D segment of the molecule. For comparison, preliminary ^2H NMR data were obtained with Z-D-Phe-L-PheGly in bilayers of perdeuterated DPPC. Only small changes in ^2H quadrupole splittings were observed for these bilayers also (data not shown).

Effects of Z-D-Phe-L-PheGly on Phospholipid Bilayers with Small Radii of Curvature. Previously it was reported that Z-D-Phe-L-PheGly inhibited the formation of small unilamellar vesicles (SUV) upon sonication of dispersions of egg PC containing the peptide (Kelsey et al., 1991). The specificity of this effect was examined using another, related hydrophobic peptide, Z-Gly-L-Phe, which shows little or no inhibition of membrane fusion (Kelsey et al., 1990). The latter peptide did not inhibit the formation of SUV. Thus, the ability of these hydrophobic peptides to inhibit the formation of small vesicles appears to exhibit a similar pattern to that observed in their inhibition of vesicle membrane fusion. It was suggested that Z-D-Phe-L-PheGly inhibited the formation of phospholipid structures with a small radius of curvature.

That study was expanded to investigate the mechanistic basis of this effect of Z-D-Phe-L-PheGly on the formation of SUV by sonication. Figure 5 shows, as a function of pH, the effect of sonication of dispersions of DPPC containing Z-D-Phe-L-PheGly at a 3:1 mole ratio (DPPC/Z-D-Phe-L-PheGly), mixed with the phospholipid when it was in organic solvent. Z-D-Phe-L-PheGly retarded the formation of SUV by sonication at pH 4.5. Above pH 6.7, SUV could be formed even in the presence of Z-D-Phe-L-PheGly.

It was reported previously that SUV of DPPC, incubated below the gel-to-liquid-crystal-phase transition temperature of DPPC, grew to sizes larger than the starting SUV (Schullery et al., 1980). Vesicles with diameters of about 70 nm were observed as the final product. Figure 6 shows this effect reproduced, using light scattering to follow the changes in vesicle structure. SUV of DPPC were formed at 50 °C and then incubated at 23 °C. Electron microscopy of the products of the incubation of pure DPPC SUV at 23 °C showed good agreement with previously published data (Schullery et al., 1980) (data not shown).

The effects of Z-D-Phe-L-PheGly on this phenomenon were then determined. DPPC SUV were formed containing Z-D-Phe-L-PheGly by increasing the pH of the medium to 7.4. As shown in Figure 6, the presence of Z-D-Phe-L-PheGly in the vesicles (incorporated prior to hydration of the lipid) caused a rapid increase in the light scattering of the sample at pH 7.4. Figure 7 shows that this increase in light scattering corresponded to the development of much larger structures, at pH 7.4, in the presence of Z-D-Phe-L-PheGly than observed over the same time of incubation for DPPC SUV formed without Z-D-Phe-L-PheGly. Figure 8 shows freeze–fracture electron micrographs taken during the course of incubation of DPPC SUV containing Z-D-Phe-L-PheGly. Immediately after sonication, the SUV of DPPC dominate the preparation, consistent with the low level of static light scattering seen in Figure 6. At 30 min, larger phospholipid assemblies are formed, and by 60 min, large sheetlike structures are formed by the phospholipids in the presence of the fusion inhibitory peptide. As shown in Figure 6, the noninhibitory (toward membrane fusion) peptide, Z-GlyPhe, did not have any significant effect on the final vesicle size, compared to the absence of peptide. Previous measurements of the partitioning of this peptide into the membrane indicated that Z-GlyPhe was incorporated into the membrane to the same extent as Z-D-Phe-L-PheGly (Kelsey et al., 1991).

This DPPC SUV system, below its gel-to-liquid-crystalline-phase transition temperature, offers a pathway for growth in vesicle size (in the liquid-crystalline state, the system is stable). On the other hand, Z-D-Phe-L-PheGly apparently favors the formation of sheetlike assemblies in place of the small vesicles (SUV). These results were consistent with a stabilization by Z-D-Phe-L-PheGly of phospholipid bilayers in structures with much larger radii of curvature. In the case of these SUV, two radii of curvature are relevant. One reflects the outer leaflet of the vesicle bilayer and is a positive radius of curvature. The other reflects the inner leaflet of the vesicle bilayer and is a negative radius of curvature.

To attempt to differentiate between affects in these two halves of the vesicle bilayer, the following experiment was performed. DPPC was sonicated in the absence of Z-D-Phe-L-PheGly. Z-D-Phe-L-PheGly was then added in methanol. In this experiment, therefore, the Z-D-Phe-L-PheGly should initially have access only to the outer leaflet of the vesicle. Control DPPC SUV were treated with the same amount of methanol. Figure 9 shows the results from this experiment. DPPC SUV did not show the same rapid growth if Z-D-Phe-L-PheGly was added after the SUV were formed. Measurement of the partitioning of the peptide, performed as described previously (Kelsey et al., 1991), indicated that there was no change in partitioning of Z-D-Phe-L-PheGly between the aqueous medium and the membrane as a function of time over which the experiments described in Figures 6 and 8 were performed. It was noted previously that addition of Z-D-Phe-L-PheGly in methanol led to the incorporation of only a

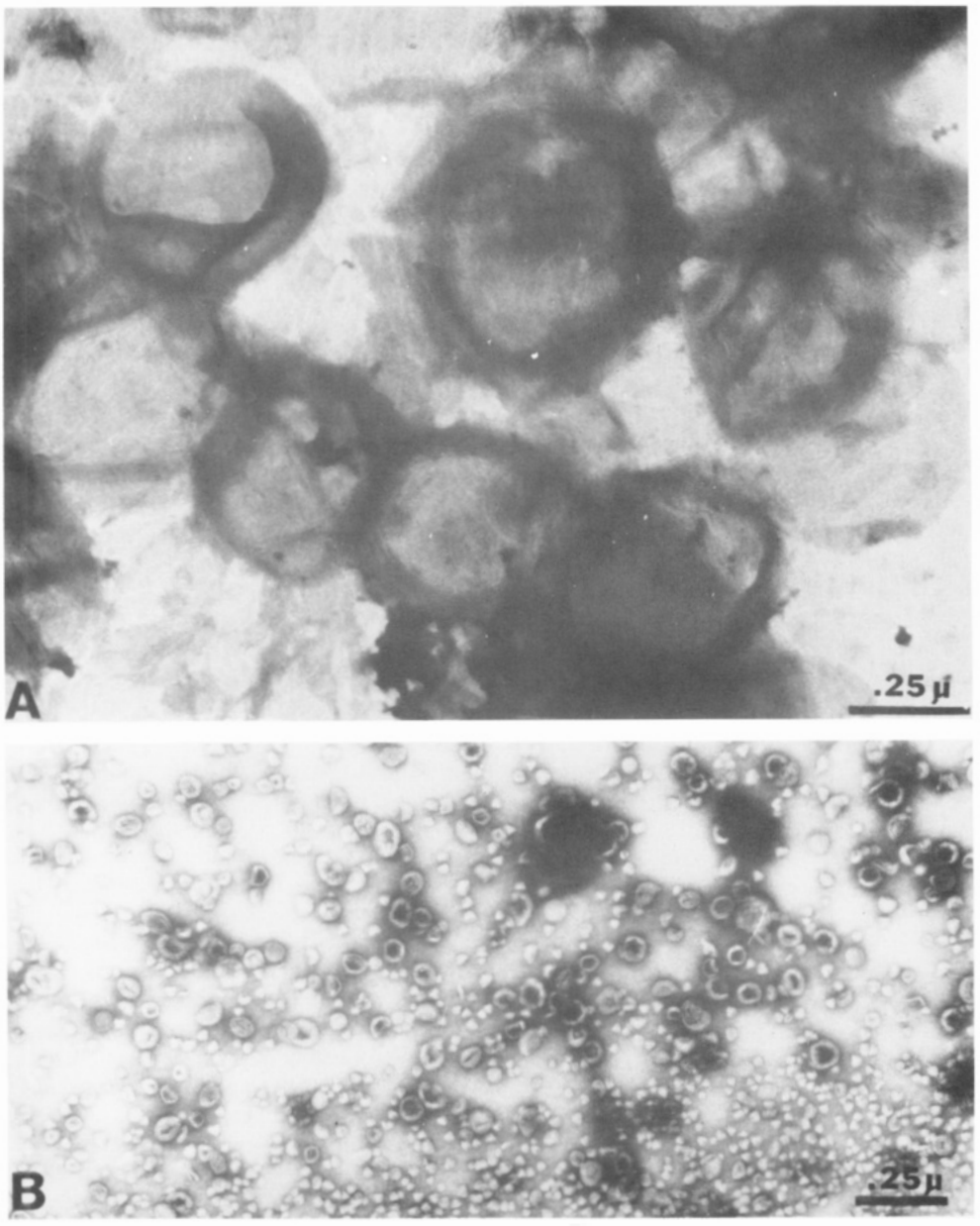

FIGURE 7: Negative-stain electron microscopy of DPPC membranes formed originally into SUV by sonication with and without Z-D-Phe-L-PheGly and then incubated as described in the text at 23 °C: (A) DPPC/Z-D-Phe-L-PheGly (3:1 mole ratio); (B) in the absence of Z-D-Phe-L-PheGly. Bar represents 0.25 μm.

portion of the peptide into the membrane. This observation was taken into account along with the exposure of only about two-thirds of the phospholipid on the outer surface of the SUV, to determine the amount of peptide to add to achieve the same peptide:phospholipid ratios attained when the peptide was mixed with the phospholipid in organic solvent prior to hydration.

Discussion

The hydrophobic peptide Z-D-Phe-L-PheGly inhibits infection by some enveloped viruses (Richardson & Choppin, 1983; Richardson et al., 1980) and does so most likely as a consequence of inhibition of membrane fusion (Kelsey et al., 1990, 1991), a required step in viral entry. The present study was designed to investigate the interaction between Z-D-Phe-L-PheGly and phospholipids to uncover some indication of how this peptide inhibits membrane fusion. It was suggested previously that the target of Z-D-Phe-L-PheGly was, at least in part, the lipid bilayer (Kelsey et al., 1991). In particular, Z-D-Phe-L-PheGly appeared to selectively perturb the structure of a putative intermediate in the fusion pathway of LUV of *N*-methyl-DOPE. The structure of this putative intermediate was characterized by an isotropic ^{31}P NMR resonance, indicating that the phospholipids defined a structure with a small radius of curvature (Ellens et al., 1989). The effect of Z-D-Phe-L-PheGly was to broaden the ^{31}P resonance from this phospholipid assembly, consistent with an increase in the radius of curvature of the assembly.

Experiments in the present study indicated that Z-D-Phe-L-PheGly had little effect on the organization of phospholipids in multilamellar liposomes (large radii of curvature). DSC of DPPC, ^{13}C NMR of the carbonyls of phosphatidylcholine, and ^{2}H NMR of phosphatidylcholine selectively deuterated in the middle of the chain showed only modest effects of Z-D-Phe-L-PheGly on the structure of the bilayers. These data suggest that Z-D-Phe-L-PheGly does not strongly perturb the motion and orientation of phospholipids in bilayers with a large radii of curvature.

In strong contrast to the interaction of Z-D-Phe-L-PheGly with relatively flat bilayers, the observed effects of Z-D-Phe-

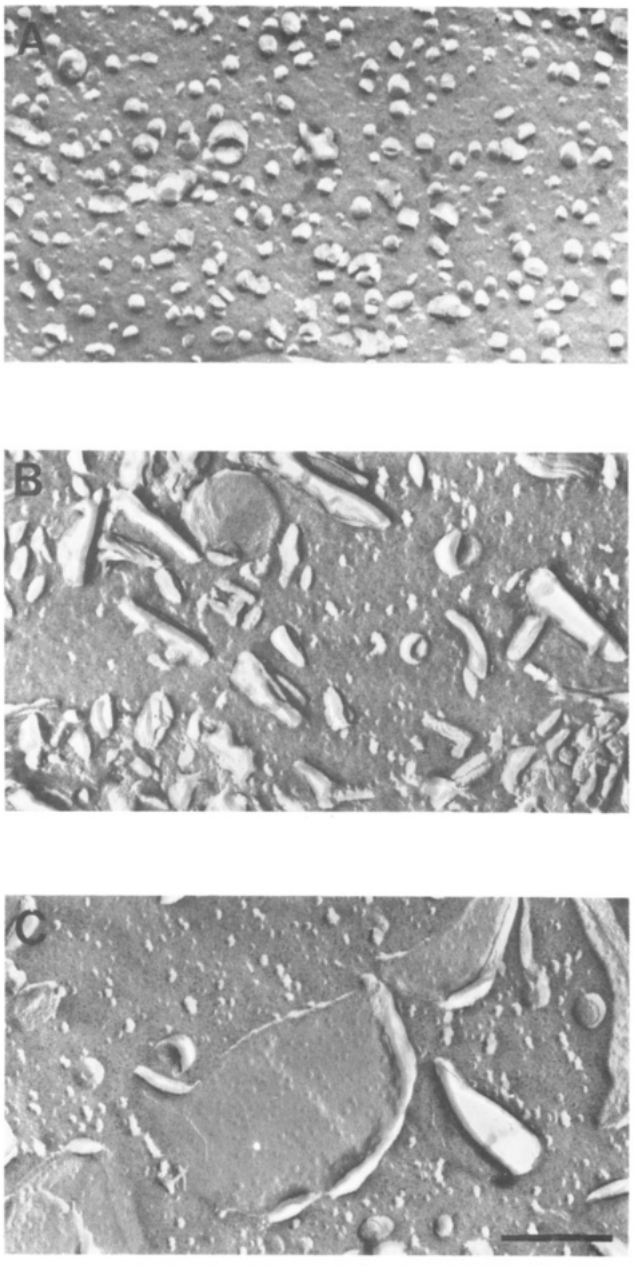

FIGURE 8: Freeze–fracture electron microscopy of DPPC membranes formed originally into SUV by sonication with Z-D-Phe-L-PheGly (DPPC/Z-D-Phe-L-PheGly 8:1 mole ratio). (A) Sample taken immediately after cessation of sonication. (B) Sample taken after 30 min of incubation at 23 °C. (C) Sample taken after 60 min of incubation at 23 °C. Bar represents 0.25 μm.

L-PheGly on SUV, or phospholipid bilayers with a small radius of curvature, indicated a significant perturbation of the organization of the phospholipid assembly. At low pH, Z-D-Phe-L-PheGly retards the formation of phosphatidylcholine (egg and dipalmitoyl) SUV by sonication. At higher pH and in the presence of Z-D-Phe-L-PheGly, DPPC forms an unstable complex with the peptide. After termination of sonication, these small complexes rapidly grew into structures with very large (nearly infinite) radii of curvature in the presence of Z-D-Phe-L-PheGly, when incubated below the gel-to-liquid-crystalline-phase transition temperature. The time course of this phenomenon was measured by light scattering, which was very sensitive to aggregation, and therefore the apparent kinetics observed were likely affected by the rate of aggregation, as was suggested previously (Schmidt et al., 1981). However, as shown by electron microscopy, the presence of Z-D-Phe-L-PheGly induced the formation of flat sheetlike assemblies

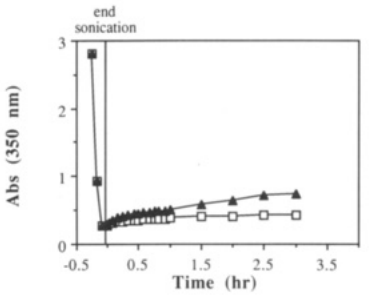

FIGURE 9: Representative experiment showing the effect of the addition of Z-D-Phe-L-PheGly (6:1 lipid:peptide molar ratio) only to the outside of the DPPC SUV immediately after sonication (as described in text). SUV were formed at 50 °C, peptide was added, and then the mixture was incubated at 23 °C. (▲) In the presence of Z-D-Phe-L-PheGly; (□) control, in the absence of Z-D-Phe-L-PheGly but with the same amount of methanol.

by the phospholipids. In the absence of Z-D-Phe-L-PheGly, the phospholipids formed vesicles only modestly larger than the starting SUV.

DPPC SUV were previously reported to increase in size when incubated below the gel-to-liquid-crystalline-phase transition temperature of the phospholipid (for SUV of DPPC, the calorimetrically observed transition centered around 37 °C) (Schullery et al., 1980). This increase in vesicle size was suggested to be a result of packing strains introduced into the phospholipid bilayer due to the small radius of curvature of the SUV and the gel state of the phospholipid. The modest growth in vesicle size for the pure phospholipid was suggested to occur until the packing strains were relieved by the increase in the radius of curvature of the phospholipid bilayer (Schullery et al., 1980).

The dramatic increase in the radius of curvature of the phospholipid bilayers in the presence of Z-D-Phe-L-PheGly (over that observed in the absence of the peptide) suggests a different mechanism. The presence of the peptide seems to release the constraint of the bilayer to form vesicles. Sheetlike assemblies, often with apparently open bilayer edges, were observed. These assemblies had much larger radii of curvature caused by the inhibitory peptide. In this DPPC SUV system, a pathway exists for particle growth to relieve the strains on packing found in the SUV bilayer below the gel-to-liquid-crystalline-phase transition. [This pathway was called fusion by previous investigators (Schullery et al., 1980), but it clearly is not the same kind of membrane fusion that is inhibited by Z-D-Phe-L-PheGly.] The presence of Z-D-Phe-L-PheGly may introduce a different packing condition into the bilayer, leading to the formation of extended sheets with an almost infinite radius of curvature of the bilayer.

By introducing Z-D-Phe-L-PheGly asymmetrically into the outer leaflet of the DPPC SUV, it was possible to test whether Z-D-Phe-L-PheGly introduced packing strains into the outer leaflet of the bilayer to induce the increase in the radius of curvature. This leaflet can be considered to exhibit a positive radius of curvature. The data in Figure 8 show little effect of Z-D-Phe-L-PheGly when it is in the outer leaflet of the SUV bilayer. Therefore, the inhibitory peptide has little effect on the positive radius of curvature of the SUV. From these data, however, one cannot completely rule out a stabilizing influence on the positive radius of curvature.

Therefore, by difference one can infer that Z-D-Phe-L-PheGly exerts its effects primarily in the inner leaflet of the vesicle bilayer with its negative radius of curvature. This conclusion may suggest that the amphipathic Z-D-Phe-L-PheGly inserts into the bilayer with its carboxyl terminal at the interface between the aqueous phase and the hydrophobic

interior of the membrane. Such a peptide might be expected to insert into the outer leaflet where packing defects between lipid headgroups might accommodate a relatively modestly sized amphipathic compound. In the inner leaflet, the relatively tight headgroup packing (Huang, 1969; Schmidt et al., 1977) may be profoundly incompatible with the addition of Z-D-Phe-L-PheGly.

This discussion implies that Z-D-Phe-L-PheGly may destabilize phospholipid bilayers with a negative radius of curvature. Other available data also suggest such an effect. Z-D-Phe-L-PheGly has been shown to stabilize the lamellar phase relative to the hexagonal II phase (Epand, 1986). Since the hexagonal II phase is characterized, in the above nomenclature, by a negative radius of curvature (Yeagle, 1987), this observation can be understood by the same model as just described.

Furthermore, structures that have been suggested to be part of the mechanism of membrane fusion are also characterized by phospholipids in an assembly with a negative radius of curvature. These include interlamellar attachments (ILA) (Hui et al., 1981a,b; Siegel et al., 1989) and intramembranous inverted micelles (Cullis & Kruijff, 1978; Siegel, 1986). Z-D-Phe-L-PheGly would, in the context of the model presented here, destabilize such structures, or inhibit their formation.

Therefore, a mechanism can be postulated for the inhibition of membrane fusion by Z-D-Phe-L-PheGly. The inhibitory peptide inhibits the formation of phospholipid assemblies that are on the pathway of membrane fusion, assemblies that are characterized by a negative radius of curvature. Inhibition of the formation of important intermediates in the fusion pathway by Z-D-Phe-L-PheGly thereby inhibits membrane fusion. This model of inhibition may also apply to viral fusion, since Z-D-Phe-L-PheGly effectively inhibits the fusion of enveloped viruses with vesicles and with cells.

Registry No. Z-D-Phe-L-PheGly, 75539-79-6; DPPC, 63-89-8; dioleoylphosphatidylcholine, 4235-95-4.

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