Thermodynamics of Interaction of the Fusion-Inhibiting Peptide Z-D-Phe-L-Phe-Gly with Dioleoylphosphatidylcholine Vesicles: Direct Calorimetric Determination†

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ABSTRACT: The binding of the fusion-inhibiting peptide Z-D-Phe-L-Phe-Gly to unilamellar lipid vesicles of dioleoylphosphatidylcholine (DOPC) was investigated by isothermal titration calorimetry (ITC). The peptide Z-D-Phe-L-Phe-Gly is known to inhibit fusion of myxo- and paramyxoviruses with cells as well as cell−cell and vesicle−vesicle fusion in model systems. Calorimetric titrations conducted over a range of temperatures permitted characterization of the thermodynamics of the interaction of Z-D-Phe-L-Phe-Gly with model DOPC lipid membranes. Simultaneous global analysis of 15 ITC binding curves acquired at four different temperatures allowed determination of the equilibrium site association constant (K), stoichiometry of binding (n), binding enthalpy change (ΔH), and heat capacity change of binding (ΔC_p) in a single set of experiments. The binding affinity and enthalpy change per mole of DOPC bound at 25 °C was log $K = 2.463 \pm 0.075$ and $\Delta H = -1.07 \pm 0.12$ kcal/mol DOPC while the binding heat capacity change per mole of DOPC bound was $\Delta C_p = -20.3 \pm 2.8$ cal/(K·mol DOPC) with a temperature dependence (from 10−45 °C) of $d(\Delta C_p)/dT = 0.37 \pm 0.18$ cal/(K²·mol DOPC). A temperature-independent binding stoichiometry was determined to be $n = 5.56 \pm 0.33$ DOPC molecules per Z-D-Phe-L-Phe-Gly. A comparison of these results with previous peptide−lipid binding studies is discussed as is their relevance to a current model of the interaction of fusion-inhibiting peptides with phospholipid membranes.

Viral membrane glycoproteins are known to regulate the penetration and viral-induced cell fusion event in the myxo- and paramyxoviruses (Klenk *et al.*, 1970; Wiley, 1985). Glycoprotein F in the paramyxoviruses and HA in the myxoviruses initiate fusion when cleaved by host cell proteases into active forms F1 and HA2 (Hsu *et al.*, 1987). The sequence of amino acids near the active N-terminal region of F1 has been shown to be similar for a number of paramyxoviruses, including Newcastle disease, Sendai, SV5, and mumps (Scheid *et al.*, 1978; Wiley, 1985; Hsu *et al.*, 1987). Sequence overlap was also observed to extend to the N-terminal region of the myxovirus HA2 fusion proteins (Scheid *et al.*, 1978). This similarity led Richardson and co-workers to synthesize a series of small peptides analogous to the F1 and HA2 N-terminal sequences. Many of these peptides were found to inhibit viral infectivity, hemolysis, and cell fusion in cultured cells (Richardson *et al.*, 1980). In particular, the tripeptide Z-D-Phe-L-Phe-Gly was found to be unusually effective in inhibiting reproduction of the Sendai, SV5, and measles viruses. When studies were extended to *in vitro* fusion of liposomes, it was found that these peptides inhibit the fusion of DOPE-Me[1] lipid vesicles (Kelsey *et al.*, 1990) and stabilize DPPC lipid monolayers with large radii of curvature (Yeagle *et al.*, 1992). These results suggest that the peptides are not merely competing for (and thus blocking) a fusion receptor on the surface of the cell, but derive effectiveness from nonspecific physical interaction with the lipid bilayer itself. As a result, it is now generally acknowledged that liposomes provide a good model system for studying the mechanism of fusion inhibition by these peptides.

Isothermal titration calorimetry (ITC) was employed to study the thermodynamics of interaction of the peptide Z-D-Phe-L-Phe-Gly with DOPC vesicles. ITC is a technique allowing not only elucidation of the affinity and stoichiometry of interaction between species in solution but also direct measurement of binding enthalpy changes (Wiseman *et al.*, 1989; Freire *et al.*, 1990; Bains & Freire, 1991; Breslauer *et al.*, 1992). Physically, ITC measures the heat of reaction associated with reactant binding as it is titrated into a solution of its reaction partner. As titration proceeds, the fractional saturation of binding sites increases until, at full saturation, no further heat is generated upon additional injection of reactant. Analysis of observed reaction heats as a function of injection number permits simultaneous elucidation of the binding stoichiometry (n), enthalpy change (ΔH), and affinity (i.e., free energy change, ΔG) of the reaction, therefore permitting definition of the binding entropy change (ΔS) as well. ITC has often been applied to ligand binding,

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[1] Abbreviations: Ala, alanine; DMPC, dimyristoylphosphatidylcholine; DOPE-Me, dioleoyl-*N*-methylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; Gly, glycine; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; Phe, phenylalanine; Trp, tryptophan; UV, ultraviolet; Z, benzyloxycarbonyl; ZFFG, Z-D-Phe-L-Phe-Gly.

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enzyme−substrate interactions, and other specific interactions to determine binding thermodynamics at a given temperature (Bolen *et al.*, 1971; Myers *et al.*, 1987; Schön & Freire, 1989; Sehl & Castellino, 1990; Cox *et al.*, 1990). By conducting the titrations as a function of temperature, the heat capacity changes of binding (ΔC_p) and its temperature dependence can also be determined, providing for a thorough thermodynamic characterization. A simultaneous global analysis approach was used to determine all of the thermodynamic parameters described above from the temperature-dependant data sets that were acquired.

Yeagle and co-workers have studied the effect of Z-D-Phe-L-Phe-Gly on the fusion behavior of phospholipid vesicles as a function of concentration (Kelsey *et al.*, 1990, 1991; Yeagle *et al.*, 1992). Its effect on the lamellar to nonlamellar phase transition of phospholipid−water systems has also been investigated (Epand *et al.*, 1987). From a theoretical perspective, Yeagle *et al.* (1992) have proposed that Z-D-Phe-L-Phe-Gly may inhibit fusion by destabilizing phospholipid monolayers with negative radii of curvature. Specifically, the monolayer on the inside of small unilamellar vesicles and some of the structures proposed to be involved in membrane fusion (Cullis & de Kruijff, 1978; Siegel, 1986; Siegel *et al.*, 1989). No quantitative determination of the thermodynamics of Z-D-Phe-L-Phe-Gly interaction with phospholipids has previously been performed, however, as has been done for other small peptides (Jain *et al.*, 1985; Jacobs & White, 1986, 1989). In our experiments, unilamellar DOPC liposomes were titrated into a solution of free peptide as a function of temperature. By combining the resulting thermodynamic data with physical data available in the literature, we show that our results add some support for the model proposed by Yeagle and co-workers (Yeagle *et al.*, 1992) for the interaction of Z-D-Phe-L-Phe-Gly with phospholipid bilayers.

MATERIALS AND METHODS

Materials. DOPC was purchased from Avanti Polar Lipids, Birmingham, AL and tested for purity by thin layer chromatography in a 90:54:5.6:5.3 $CHCl_3$/MeOH/25% NH_4OH/H_2O solution and used without further purification. Z-D-Phe-L-Phe-Gly was purchased from Sigma Chemical Co. (St. Louis, MO) and used as received. A 20 mM sodium phosphate buffer at pH 7.2 was used throughout. Water was deionized and filtered and had a typical resistivity of 15 MΩ·cm. All solvents were of reagent grade or better.

Sample Preparation. Peptide was dissolved in buffer to a concentration of 1 mM by gentle warming. At this concentration, light scattering using a Coulter N4 light scattering instrument (Coulter Electronics, Inc., Miami Lakes, FL) showed no evidence of peptide aggregation. Lipid vesicle formation was begun by placing between 300 and 500 mg of DOPC powder in a large test tube and dissolving it into several milliliters of chloroform. The bulk of the chloroform was removed with a stream of dry argon. The test tube was placed under vacuum (10^{-3} torr) for at least 5 h to remove the remaining chloroform, leaving a film of lipid on the test tube walls. Buffer was added to make a 100 mg/mL concentration of lipid, and the test tube was vortexed to hydrate the lipid film. The mixture was frozen on dry ice for 10 min, reheated to room temperature, and vortexed again. This procedure was repeated three times to ensure adequate hydration of the lipid. Next, the lipid suspension was extruded to produce unilamellar lipid vesicles. Extrusions were performed at room temperature using a LIPEX extruder (Vancouver, BC) with Nucleopore polycarbonate membranes (Mayer *et al.*, 1986). The lipid suspension was passed through a series of filter membrane pore sizes: two times through a 0.6 μm filter, three times through two stacked 0.08 μm filters, and four times through two stacked 0.05 μm filters. The resulting unilamellar vesicles were sized using the Coulter N4 light scattering instrument to determine final vesicle size and distribution. Vesicles used in these experiments were sized to 600 ± 100 Å. Finally, 200 μL of vesicle suspension was removed to preweighed test tubes and lyophilized to determine final lipid concentration by assuming that the mass of resulting lyophilized pellets was due only to remaining 20 mM sodium phosphate buffer and lipid.

Isothermal Titration Calorimetry. Isothermal titration calorimetry was performed using an Omega instruments calorimeter (Microcal, Amherst, MA) described elsewhere (Wiseman *et al.*, 1989). Two different Omega ITCs were employed in these studies, one optimized for better performance at lower temperatures (10 and 25 °C), the other at higher temperatures (37 and 45 °C). Calorimeter sample chambers, possessing effective sample volumes of 1.3625 mL (10 and 25 °C) and 1.388 mL (37 and 45 °C), were loaded to overfill conditions with ~1 mM Z-D-Phe-L-Phe-Gly. A series of 10 μL injections, ranging in number from 13−52 injections per experiment, of ~100 mM DOPC were titrated into continuously stirred solutions of peptide at 5 min intervals. Three experiments each were performed at 10 and 25 °C, four at 37 °C, and five at 45 °C, for a total of 15 independent titrations.

Data Analysis. All 15 data sets were considered simultaneously by a global nonlinear least-squares analytical strategy (Straume, 1994) in which DOPC molecules were assumed to associate with multiple independent and thermodynamically identical lipid binding sites on Z-D-Phe-L-Phe-Gly. Individual observed heat events (i.e., the measured heats associated with each injection of DOPC into Z-D-Phe-L-Phe-Gly) were variably weighted in analysis by estimated standard deviations corresponding to the square roots of measured heats (constrained to be greater than or equal to 1 μcal/mL of effective cell volume, an empirically determined reasonable minimum error estimate for these titration calorimeters). Although perhaps conservative, this variable weighting scheme provides a mechanism for approximately accounting for variations in measurement precision that are proportional to magnitudes of individual measured heats (i.e., larger absolute heats per injection have proportionately larger associated measurement uncertainties).

Individual heats per injection, q_j, were defined as

$$q_j = nV\Delta H[\mathrm{P}]_j(F_j - F_{j-1}) \quad (1)$$

where j is injection number, n is the number of (independent and thermodynamically identical) DOPC binding sites per Z-D-Phe-L-Phe-Gly, V is the effective volume of peptide solution being titrated (i.e., the calorimeter cell volume), ΔH is the enthalpy change for binding a DOPC molecule to a site on Z-D-Phe-L-Phe-Gly, $[\mathrm{P}]_j$ is the concentration of peptide after the jth injection, and F_j and F_{j-1} correspond to the fractional occupancy of DOPC binding sites on Z-D-Phe-L-Phe-Gly after j and $j-1$ injections, respectively. It should

be noted that the product $n[P]_j(F_j - F_{j-1})$ is simply the concentration of lipid that was bound to peptide binding sites during injection j.

The temperature-dependent binding enthalpy change, ΔH, was defined as

$$\Delta H = \Delta H_{ref} + [\Delta C_{p0} + \Delta C_{p1}(T - T_{ref})](T - T_{ref}) \quad (2)$$

where ΔH_{ref} is the binding enthalpy change (per mole of DOPC bound) at the reference temperature, T_{ref} (taken as 298.15 K), and ΔC_{p0} and ΔC_{p1} are the binding heat capacity change at the reference temperature and its linear temperature dependence, respectively.

Fractional binding, F_j, can be determined from the site association (site binding) constant, K, and lipid concentration, $[L]_j$, to be

$$F_j = \frac{K[L]_j}{1 + K[L]_j} \quad (3)$$

The site association constant, K, is given by the following relationship

$$K = \exp(-\Delta G/RT) \quad (4)$$

where the free energy change for binding, ΔG, is defined as

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

In accordance with the above definitions, the temperature-dependent binding entropy change can be calculated to be

$$\Delta S = \Delta S_{ref} + [\Delta C_{p0} + \Delta C_{p1}(T - T_{ref})] \ln(T/T_{ref}) \quad (6)$$

where ΔS_{ref} is the binding entropy change at the reference temperature.

During analysis, the free energy change of binding at the reference temperature, ΔG_{ref}, was considered in terms of the fitting parameter log K_{ref} as

$$\Delta G_{ref} = -RT_{ref} \ln(10^{\log K_{ref}}) \quad (7)$$

The entropy change of binding at the reference temperature, ΔS_{ref}, was calculated relative to T_{ref} and the fitting parameter ΔH_{ref} as

$$\Delta S_{ref} = \frac{\Delta H_{ref} - \Delta G_{ref}}{T_{ref}} \quad (8)$$

Maximum likelihood parameter values and approximate nonlinear joint parameter confidence limits were evaluated by a modified Gauss–Newton nonlinear least-squares minimization algorithm described previously (Johnson & Frasier, 1985; Straume *et al.*, 1991). All parameter uncertainties were evaluated at 68.26% confidence probability (corresponding to the probability of one standard deviation of a Gaussian distribution) and reported as approximate standard deviations calculated as one-half the difference between derived upper and lower 68.26% confidence limits.

RESULTS

Control titrations of DOPC vesicle suspensions into buffer alone (i.e., containing no Z-D-Phe-L-Phe-Gly) consistently produced only small-magnitude reference heats (i.e., heats of dilution) that ranged from approximately −5 to 15−20 μcal per injection (data not shown). Reference heats thus represented a fraction of a percent to only a few percent of the maximally observed experimental heats and were subtracted from experimental calorimetric titration data prior to analysis.

Fifteen calorimetric titrations of 13−52 10 μL injections of ~100 mM DOPC into ~1.4 mL of ~1 mM Z-D-Phe-L-Phe-Gly were performed at 9.8−10 °C (n = 3), 25 °C (n = 3), 37 °C (n = 4), and 43.1−45.5 °C (n = 5). Both global and sequential fits to the titration data were carried out, as described below. Before fits were made, however, the DOPC concentration was modified to truly reflect the fraction of lipid available to interact with the peptide. Yeagle *et al.* (1992) have shown that Z-D-Phe-L-Phe-Gly does not permeate across a phospholipid bilayer, so only the lipid fraction in the outer monolayer was considered available for interaction with the peptide. The ratio of outer to inner vesicle surface area yields the fraction of lipid in the outer monolayer $f_o = r_o^2/(r_o^2 + r_i^2)$, where r_o is the outer vesicle radius and r_i is the inner vesicle radius. Assuming a mean vesicle radius of r_o = 300 Å (diameter = 600 Å) and a DOPC bilayer thickness of 63 Å (Gruner *et al.*, 1988), we find r_i = (300 − 63) = 237 Å and f_o = ~0.616. In all data analysis, therefore, the lipid concentration used was the effective concentration, $[DOPC]_{eff}$, which was calculated from the total DOPC concentration, $[DOPC]_{tot}$, as: $[DOPC]_{eff} = f_o[DOPC]_{tot}$.

All 15 titration data sets were analyzed simultaneously by a global analytical protocol in which optimal parameter values for DOPC-to-Z-D-Phe-L-Phe-Gly binding stoichiometry (n), affinity (as log K), enthalpy change (ΔH), and heat capacity change (ΔC_p) were sought. In addition, an examination for any temperature dependence of binding stoichiometry and/or heat capacity change was conducted. Accommodation was also made for effects of imperfect reference heat correction. Simultaneous analysis of the 15 independently acquired data sets was performed using variably weighted values of observed heats (see *Data Analysis* under Materials and Methods) as a function of injection number, the primary independent variable. Six secondary independent variables were also considered during the analysis: (i) the starting concentration of Z-D-Phe-L-Phe-Gly being titrated, (ii) the effective concentration of DOPC being titrated, (iii) the volume of the titration cell, (iv) the volume of DOPC vesicle suspension introduced per injection, (v) the temperature, and (vi) the particular calorimetric titration experiment (by way of a dummy identifier variable).

Results of the simultaneous global analysis are presented in Table 1. Results of analyses in which reference heat corrections to experimental calorimetric titration data were assumed correct are tabulated in the first group of four rows in Table 1 (indicated with the notation q_c's = 0; see below for an explanation of q_c). The first row contains results from analysis in which neither the binding stoichiometry nor the heat capacity change was permitted to exhibit temperature dependence. The second and third rows present results from analyses in which a linear temperature dependence was modeled for either the binding stoichiometry [implemented as $n = n_0 + n_1(T - T_{ref})$; second row] or the binding heat capacity change (see eq 2; third row). The fourth row shows results of analysis when both the binding stoichiometry and heat capacity change were free to exhibit linear temperature dependence.

Table 1: Results of Simultaneous Global Analysis of Calorimetric Titrations[a]

	n_0 (DOPC/ZFFG)	n_1 (DOPC/K-ZFFG)	log K_{ref}	ΔH_{ref} (kcal/mol DOPC)	ΔC_{p0} (cal/K·mol DOPC)	ΔC_{p1} (cal/K²·mol DOPC)	σ_{fit}^2 (NDF)
q_c's = 0	6.00 (0.21)	[0]	2.666 (0.027)	−0.779 (0.034)	−13.45 (0.74)	[0]	1.58 (492)
	6.13 (0.25)	−0.018 (0.013)	2.665 (0.031)	−0.772 (0.038)	−15.4 (1.5)	[0]	1.55 (491)
	6.02 (0.22)	[0]	2.673 (0.028)	−0.812 (0.039)	−14.04 (0.83)	0.199 (0.067)	1.44 (491)
	6.07 (0.26)	−0.006 (0.013)	2.672 (0.029)	−0.807 (0.044)	−14.6 (1.6)	0.187 (0.068)	1.44 (490)
q_c's ≠ 0	5.42 (0.40)	[0]	2.415 (0.075)	−1.07 (0.13)	−21.4 (3.4)	[0]	0.947 (477)
	5.42 (0.43)	−0.024 (0.029)	2.390 (0.071)	−1.12 (0.14)	−25.9 (4.2)	[0]	0.932 (476)
	5.56 (0.33)	[0]	2.463 (0.075)	−1.07 (0.12)	−20.3 (2.8)	0.37 (0.18)	0.821 (476)
	5.57 (0.33)	−0.002 (0.022)	2.463 (0.077)	−1.07 (0.12)	−20.4 (2.8)	0.37 (0.19)	0.823 (475)

[a] n_0, lipid-to-peptide binding stoichiometry at 25 °C; n_1, linear temperature dependence of lipid-to-peptide binding stoichiometry; log K_{ref}, base-10 logarithm of equilibrium lipid-to-peptide site association constant at 25 °C; ΔH_{ref}, lipid-to-peptide binding enthalpy change at 25 °C; ΔC_{p0}, lipid-to-peptide binding heat capacity change at 25 °C; ΔC_{p1}, linear temperature dependence of lipid-to-peptide binding heat capacity change; σ_{fit}^2, weighted variance of global fit; NDF, number of degrees of freedom of fit (number of total data points minus number of parameters estimated); q_c's = 0, analyses assuming that corrections to calorimetric titration data for reference heats of vesicle dilution are correct; q_c's ≠ 0, analyses in which 15 local variables, one for each individually acquired calorimetric titration, were estimated simultaneously with global thermodynamic system parameters to account for potentially imperfect corrections by reference heats of vesicle dilution; ZFFG, the fusion inhibiting peptide Z-D-Phe-L-Phe-Gly (see text for details).

In an attempt to compensate for the effect of imperfect and imprecisely known reference heat corrections, the same four types of analyses mentioned above also were performed while considering one additional local variable for each of the 15 independent data sets. These 15 additional variables, referred to as q_c's (correction heats), represent the values to which injection heats for individual calorimetric titrations were allowed to asymptotically approach at full saturation. The four global fits described in the previous paragraph constrained each calorimetric titration curve to go to zero at full saturation which is equivalent to setting all q_c's to zero during the fit. When allowed to float during the fits, estimated q_c's converged to values in the range from −5 to 25 μcal. These values are in good agreement with the range of reference heats measured during control injections of DOPC into buffer. Results of these analyses are tabulated in the second group of four rows in Table 1 (indicated with the notation q_c's ≠ 0).

Significance probabilities that resulting variances of fit, σ_{fit}^2, represented improved characterizations of the data set by corresponding models were analyzed via Fisher's F-distribution through the test of additional terms (Bevington, 1969, pp 200−201). For example, the probability that the inclusion of a temperature dependence in the binding stoichiometry via the parameter n_1 produced an improved quality of fit in the q_c's = 0 case (i.e., the first two rows of Table 1) is calculated by the relationship

$$\frac{\sigma^2_{\text{without}}}{\sigma^2_{\text{with}}} = 1 + \left[\frac{n_{\text{additional parms}}}{N_{\text{pnts}} - n_{\text{total parms}}}\right] F(n_{\text{additional parms}}, N_{\text{pnts}} - n_{\text{total parms}}, 1 - P) \quad (9)$$

Here, σ^2_{with} and $\sigma^2_{\text{without}}$ correspond to variances of fit obtained from analyses with and without the parameter n_1 considered in the model, respectively (i.e., 1.55 and 1.58 in Table 1, respectively), $n_{\text{additional parms}}$ is the number of additional parameters being considered in the comparison (in this case, one), N_{pnts} is the total number of data points being analyzed (496), $n_{\text{total parms}}$ is the total number of parameters considered in the higher-order model (in this case, five), and the value of F is that of Fisher's F-distribution at $n_{\text{additional parms}}$ and $N_{\text{pnts}} - n_{\text{total parms}}$ numbers of degrees of freedom with significance probability P. F is calculated as the incomplete β function (Press *et al.*, 1989, pp 166−169) allowing identification of P by iterative solution of the above expression. P describes the fraction of random data sets which would provide a better fit than the new fitting function. The smaller the value of P, therefore, the more confidence in the fit. For this example, $P(n_1) = 0.0013$, indicating that inclusion of the binding site temperature dependance, n_1, in the model produced a moderate improvement in the quality of fit. Including the effect of a temperature-dependent heat capacity change of binding, ΔC_{p1}, however, was much more significant [$P(\Delta C_{p1}) < 0.000005$]. No significant additional increase in the quality of fit was obtained when n_1 was considered in addition to ΔC_{p1} in the model [$P(\Delta C_{p1} \rightarrow n_1, \Delta C_{p1}) = 0.45$]. [Note: All calculated probabilities made use of variances of fit recorded to five significant figures.] Relaxation of the correction heat constraint (i.e., on going from the q_c's = 0 to the q_c's ≠ 0 case) produced significant improvements in quality of fit ($P < 0.000005$ for all four analyses). Again in this case, however, inclusion of a term for a temperature-dependent binding heat capacity change, ΔC_{p1}, emerged as the most significant term for improving the quality of fit [$P(\Delta C_{p1}) < 0.000005$, $P(n_1) = 0.0047$, and $P(\Delta C_{p1} \rightarrow n_1, \Delta C_{p1}) > 0.999995$]. Statistically, therefore, the most probable thermodynamic description of the binding energetics of Z-D-Phe-L-Phe-Gly with DOPC vesicles considered under the conditions of these experiments is given by the seventh row of Table 1. Figure 1 shows a plot of all the experimental titration data along with the results of the most probable global fit to the data.

For completeness, individual sequential analysis of the 15 titration data sets also was carried out. Each of the 15 ITC calorimetric profiles was independently evaluated by weighted nonlinear least-squares to determine best-fit parameter values for n, log K, ΔH, and q_c, along with their associated estimated uncertainties. All 15 of the data sets produced convergence to least-squares minimum estimates for these four parameters. However, only 14 permitted successful evaluation of joint parameter confidence limits. One of the data sets at 45 °C was sufficiently poorly determined that a large degree of correlation among parameters was produced, thus not permitting estimation of parameter value uncertainty. The results from this data set were therefore excluded from further consideration in sequential analysis. The individual sequential analysis results for each titration are presented in Table

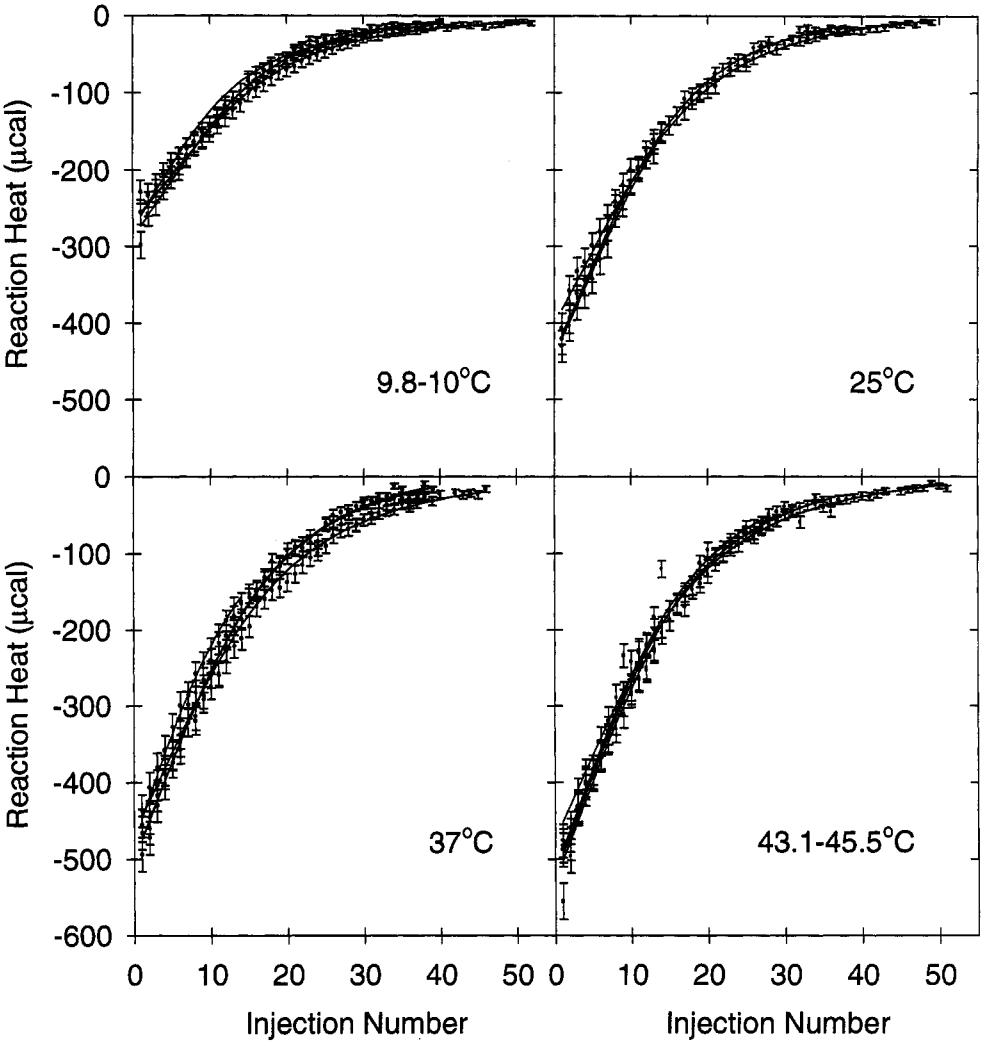

FIGURE 1: Graphic depiction of observed reaction heats versus injection number for the 15 independently acquired calorimetric titration data sets (with variable weighting indicated by associated error bars) together with best-fit curves corresponding to the best-fit parameter values of row seven in Table 1. Three data sets each are shown for 9.8−10 and 25 °C, four data sets for 37 °C, and five data sets for 43.1−45.5 °C.

Table 2: Results of Sequential Analysis of Calorimetric Titrations[a]

log K_{ref}	ΔH_{ref} (kcal/mol DOPC)	ΔC_{p0} (cal/K·mol DOPC)	ΔC_{p1} (cal/K²·mol DOPC)	σ_{fit}^2	NDF	P
2.516 (0.052)	−0.890 (0.064)	−24.1 (4.5)	[0]	1.71	25	
2.517 (0.057)	−1.05 (0.17)	−24.4 (4.7)	0.76 (0.72)	1.48	24	0.06274

n_0 (DOPC/ZFFG)	n_1 (DOPC/K-ZFFG)	σ_{fit}^2	NDF	P
5.79 (0.25)	[0]	6.63	13	
5.87 (0.38)	−0.018 (0.028)	6.64	12	>0.999995

[a] P, level of significance probability that the higher order sequential analysis, in which temperature dependence is explicitly modeled, represents a statistically significant improvement in quantitative characterization of the corresponding variably weighted data by way of the test of additional terms (see text for details); other parameters are as defined in the legend to Table 1.

2. Figure 2 shows a graphical comparison of the sequential and global analysis results which will be further described below.

The points with error bars depicted in Figure 2 represent the results of these 14 independent analyses. Corresponding values for q_c ranged from −4.7 to 29.9 μcal, in quite reasonable agreement with values to be expected based on results of control titrations of DOPC into buffer alone. Simultaneous weighted nonlinear least squares analysis of the apparent temperature dependence of ΔH and log K values was used to determine a constant heat capacity change of DOPC binding to Z-D-Phe-L-Phe-Gly, ΔC_{p0}, evaluated to be −24.1 ± 4.5 cal/(K·mol DOPC) (Table 2, first row, and the thin solid lines in the top and middle panels of Figure 2). Accommodation of a temperature dependence in the heat capacity change, ΔC_{p1}, by analysis of the temperature dependence of weighted ΔH and log K values produced a marginally statistically significant ($P = 0.063$) improvement in quality of sequential fit, but to a lesser degree of determination (i.e., with larger estimated parameter uncertainty) than that obtained from simultaneous global analysis [i.e., ΔC_{p0}(sequential) = −24.4 ± 4.7 versus ΔC_{p0}(global) = −20.3 ± 2.8 cal/(K·mol DOPC) at 298.15 K and ΔC_{p1}(sequential) = 0.76 ± 0.72 versus ΔC_{p1}(global) = 0.37 ±

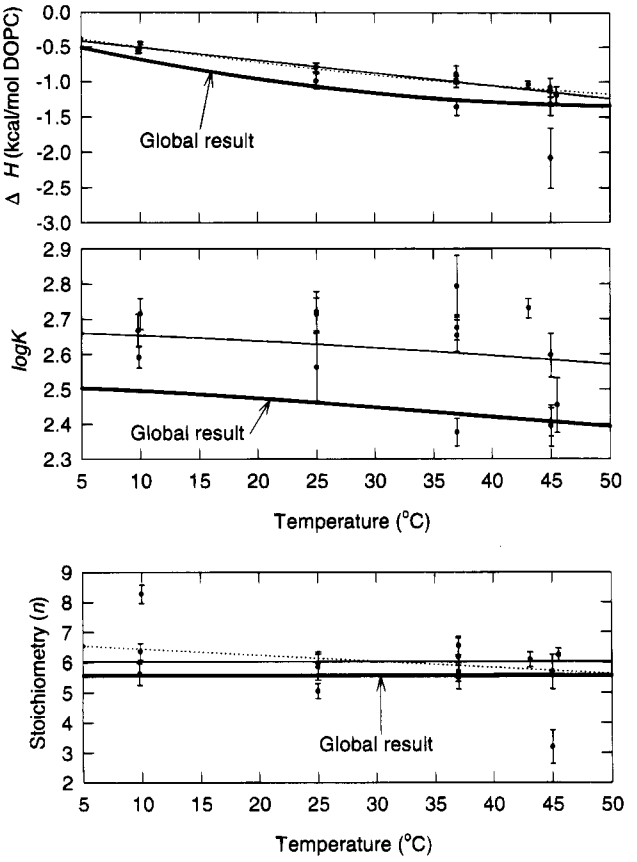

FIGURE 2: Points with error bars correspond to parameter values and estimated uncertainties obtained from independent analyses of 14 of the 15 calorimetric titration data sets obtained in this study. (One of the data sets at 45 °C was sufficiently poorly determined that independent analysis failed to permit estimation of parameter confidence limits. It was thus excluded from consideration in sequential analysis.) Thin solid lines, sequentially determined best fits excluding temperature dependence of ΔC_p (for ΔH and log K, top and middle panels) and of n (bottom panel); dotted lines, sequentially determined best fits when explicit temperature dependencies where considered for ΔC_p (for ΔH and log K, top and middle panels) and for n (bottom panel); bold solid lines, results of simultaneous global analysis.

0.18 cal/(K^2·mol DOPC)]. The second row of Table 2 and the dotted lines in the top and middle panels of Figure 2 represent results of this latter sequential analysis. The bold solid lines in the top and middle panels of Figure 2 depict the temperature dependence of ΔH and log K as given by the best-fit results from simultaneous global analysis (i.e., the results presented in Table 1, row seven). Determination of DOPC-to-Z-D-Phe-L-Phe-Gly binding stoichiometry from subsequent weighted nonlinear least-squares analysis of individually derived values for n failed, statistically, to suggest any significant temperature dependence, in agreement with the result from simultaneous global analysis (Table 2, bottom, and bottom panel of Figure 2).

The qualitative agreement between the results obtained from simultaneous global analysis and sequential analysis of intermediate results is satisfying. However, the statistically most well-determined quantitative characterization of the thermodynamics of Z-D-Phe-L-Phe-Gly interaction with DOPC vesicles is given by the results of simultaneous global analysis of the composite set of multitemperature calorimetric titration data. Tabulating these best-fit results as a function of temperature in terms of either per mole of DOPC bound or per mole of Z-D-Phe-L-Phe-Gly bound produces the thermodynamic parameter values presented in Table 3.

DISCUSSION

Numerous experimental methods, such as fluorescence spectroscopy, radiolabeling, UV spectroscopy, NMR spectroscopy, etc., historically have been applied in attempts to elucidate the binding characteristics of small molecules to lipid vesicles and micelles [Deber & Behnam, 1984; Surewicz & Epand, 1984; Jacobs & White, 1986, 1989; Lee *et al.*, 1989; Beschiaschvili & Seelig, 1990; de Kroon *et al.*, 1991; see also Chen *et al.* (1978)]. Under the best circumstances, these various techniques are capable of determining both binding affinity (K) and stoichiometry (n); however, often only the ratio of affinity to stoichiometry (K/n) is reported because independent evaluation of both parameters has often proven unreliable (Bashford *et al.*, 1979). The present study demonstrates that calorimetric titrations conducted over a range of temperatures, when analyzed by simultaneous global analysis, were capable of providing a complete thermodynamic characterization of the interaction of Z-D-Phe-L-Phe-Gly with DOPC vesicles in the temperature regime studied.

Multiple experiments were conducted at each of four temperatures to provide a high level of system determination for this relatively weak-binding interaction. Optimal conditions for extracting estimates of n, log K, and ΔH from individual calorimetric titrations are obtained when (i) the dimensionless product of the concentration of sites being titrated and the equilibrium site association constant (nK[P], in the present case) is in the vicinity of approximately 10–500 and (ii) the titration is carried out to high fractional binding site saturation with a "large" number of observations (e.g., tens of injections over which an approximately 0.1–0.9 range in fractional saturation is experienced by the system) (Wiseman *et al.*, 1989; Breslauer *et al.*, 1992). Whereas the former condition was not closely approached by the DOPC/Z-D-Phe-L-Phe-Gly system under the experimental conditions considered here (nK[P] = ~1.6), quite a broad range of fractional saturation was surveyed with calorimetric titrations involving more than ~30 injections.

Implementation of simultaneous global analysis permitted a statistically robust characterization of the binding thermodynamics of this system (see results presented in Table 1). The statistical superiority of simultaneous global analysis over sequential analysis of individual titration profiles to extract temperature dependent thermodynamic parameters has recently been discussed in the literature (Straume, 1994). It is particularly true in the case of (relatively) poorly determined individual calorimetric titrations of a low affinity interaction, as in the present case. This is because poor determination of individual titration experiments will propagate into subsequent analysis of intermediate results permitting less reliable quantitation of system properties. Additionally, simultaneous global analysis offers the advantage of retaining, during the parameter estimation process, information pertaining to parameter correlation, thus permitting more reliable parameter value confidence interval estimation than that possible from a sequential analysis (Straume, 1994).

Yeagle and co-workers have suggested that the simplest model of the interaction of Z-D-Phe-L-Phe-Gly with a lipid

Table 3: Tabulation of Thermodynamic Binding Parameters as a Function of Temperature in Terms of per Mole DOPC Bound and per Mole Z-D-Phe-L-Phe-Gly Bound[a]

	parameter	10 °C	25 °C	37 °C	45 °C
per mole DOPC bound	log K	2.496	2.463	2.430	2.408
	ΔG (kcal/mol)	−3.23	−3.36	−3.45	−3.50
	ΔH (kcal/mol)	−0.680	−1.07	−1.26	−1.32
	ΔS (cal/K•mol)	9.02	7.69	7.06	6.86
	ΔC_p (cal/K•mol)	−25.9	−20.3	−15.8	−12.8
per mole ZFFG bound	log K	13.89	13.70	13.52	13.40
	ΔG (kcal/mol)	−18.0	−18.7	−19.2	−19.5
	ΔH (kcal/mol)	−3.78	−5.94	−6.99	−7.36
	ΔS (cal/K•mol)	50.2	42.8	39.3	38.1
	ΔC_p (cal/K•mol)	−144	−113	−87.8	−71.2

[a] ZFFG, Z-D-Phe-L-Phe-Gly.

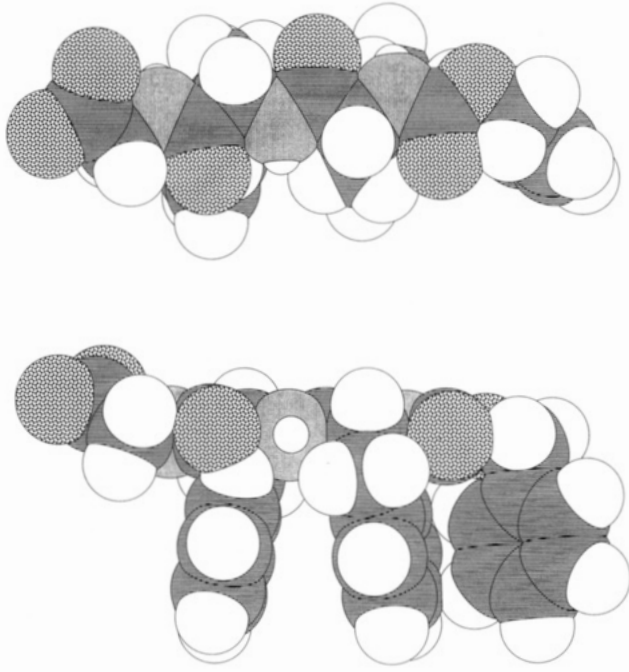

FIGURE 3: Proposed conformation for Z-D-Phe-L-Phe-Gly in association with a lipid bilayer. Dark gray, carbon; light gray, nitrogen; dotted, oxygen; white, hydrogen; small white, lone pair electron. The carboxyl terminus and amide backbone are expected to be situated at the lipid−water interfacial region while the hydrophobic phenyl ring side chains penetrate into the hydrophobic region of the bilayer. The upper figure depicts the view when looking down onto the bilayer plane while the lower figure is a cross-section view. (Figure generated by Dr. Brian Peek.)

bilayer is for the peptide to expose its hydrophilic carboxyl terminus at the lipid−water interface while burying its hydrophobic portion into the bilayer (Kelsey *et al.*, 1991; Yeagle *et al.*, 1992). The statistically most probable conformation consistent with their model is shown in Figure 3. The carboxyl terminus and the amide backbone region of the peptide partition near the lipid−water interface whereas the hydrophobic phenyl ring side chains intercalate into the hydrophobic core of the bilayer. The structure in the upper panel of Figure 3 shows a view of the peptide when looking down onto the plane of the bilayer. The perimeter of this (hydrophilic) view of the peptide is approximately 42 Å. Given an area per DOPC molecule of ~70 Å^2 (Gruner *et al.*, 1988), approximately five DOPC molecules would be expected to interact around the periphery of such a vesicle-bound peptide assuming packing on a simple square lattice near the peptide. The potential for additional interaction beneath the surface of this view of the peptide (see the side view in the bottom panel of Figure 3 for evident voids among the intercalating hydrophobic structures) and other probable conformations makes this a minimal estimate of the number of DOPC molecules that might associate with the peptide. The stoichiometry of ~5.5 DOPC per Z-D-Phe-L-Phe-Gly determined from analysis of the calorimetric titration data is remarkably consistent with this prediction based on simple steric arguments.

The free energy change of binding obtained for Z-D-Phe-L-Phe-Gly interacting with DOPC vesicles can be compared with values reported previously for small peptides interacting with lipids. Jacobs and White (1986, 1989) measured partition coefficients for a series of hydrophobic tripeptides into DMPC vesicles. They defined partition coefficients as

$$K_p = \frac{N_{bilayer}}{N_{aqueous}} \tag{10}$$

where $N_{bilayer}$ and $N_{aqueous}$ are mole fractions of bilayer-bound and aqueous peptide, respectively, as given by

$$N_{bilayer} = \frac{\text{(moles of DMPC bound peptide)}}{\text{(moles of DMPC bound peptide)} + \text{(moles of DMPC)}}$$

$$N_{aqueous} = \frac{\text{(moles of aqueous peptide)}}{\text{(moles of aqueous peptide)} + \text{(moles of water)}} \tag{11}$$

Under the conditions of their experiments (1−5 mM peptide and 1−5 mM small, unilamellar, extensively sonicated DMPC vesicles at 33 °C), Jacobs and White (1986) obtained partition coefficients ranging from 149 for Ala-Gly-Ala-*O-tert*-butyl to 3960 for Ala-Trp-Ala-*O-tert*-butyl, consistent with the greater lipid partitioning anticipated for peptides of increasing hydrophobicity (Jacobs & White, 1989). Conversion of the thermodynamic binding parameters obtained in the present study to a value for a partition coefficient expected at conditions of 3 mM peptide and 3 mM DOPC at 33 °C produced $K_{p,expected}$ = 1668 for the Z-D-Phe-L-Phe-Gly interaction with the outer monolayer of 600 Å diameter small unilamellar extruded DOPC vesicles. The value of K_p varies with concentration when using this definition so our calculated values of $K_{p,expected}$ ranged from 1009 at 1 mM DOPC and 5 mM Z-D-Phe-L-Phe-Gly to 4266 at 5 mM DOPC and 1 mM Z-D-Phe-L-Phe-Gly. Thus, even though the calculated hydrophobic molecular surface area of Z-D-Phe-L-Phe-Gly is greater than that of Ala-Trp-Ala-*O-tert*-butyl [~750 versus ~650 Å^2, respectively, as calcu-

lated from Rose *et al.* (1985)], the deduced partition coefficients (1009−4266 versus 3960, respectively) do not bear out an expected increase in hydrophobic partitioning of Z-D-Phe-L-Phe-Gly relative to Ala-Trp-Ala-*O-tert*-butyl. According to Nozaki and Tanford (1971), the free energy change of transfer from water to oil due to the hydrophobic effect, ΔG_{hyd}, is proportional to exposed surface area, A, as

$$\Delta G_{hyd} = C_s A \tag{12}$$

through a proportionality constant, C_s. Taking C_s to be −22 cal/(mol·Å^2) (Jacobs & White, 1986), Z-D-Phe-L-Phe-Gly would be expected to partition more favorably than Ala-Trp-Ala-*O-tert*-butyl by over 2 kcal/mol of free energy, a difference not reflected in respective values of estimated partition coefficients.

A number of possible explanations for this lack of an apparent effect on partition coefficient can be suggested on the basis of differences between the two experimental settings. Whereas the experimental protocol of Jacobs and White (1986) employed cosonication of mixtures of peptide and DMPC prior to evaluation of peptide partitioning, the present study involved the more "passive" approach of characterizing the interaction of preformed DOPC vesicles with peptide in solution. Therefore, we measured the interaction of peptide with only the outer, positively curved lipid surface of the vesicle while Jacobs and White's peptide interacted with both positive and negatively curved lipid surfaces. Another notable difference is the use of the saturated acyl chain lipid DMPC by Jacobs and White (1986) as opposed to the use of monounsaturated acyl chain DOPC lipids in the present study. Additionally, the small unilamellar vesicles prepared by probe sonication in the studies of Jacobs and White (1986) [as prepared by the method of Huang and Thompson (1978)] are expected to yield primarily vesicles of ~200−250 Å in diameter, considerably smaller (and thus under the influence of considerably different radius of curvature strain effects) than the ~600 Å diameter vesicles produced by the extrusion procedure employed in the present study. Finally, another factor may be related to the charge of the peptides. Z-D-Phe-L-Phe-Gly presents an exposed negatively charged carboxylic acid group whereas the Ala-X-Ala-*O-tert*-butyl series of peptides present positively charged exposed amino termini.

Another difference in conclusions drawn regarding lipid−peptide interactions between the work of Jacobs and White (1986, 1989) and that reported here has to do with the magnitude of binding enthalpy changes. Whereas Jacobs and White (1986, 1989) concluded that there was effectively no enthalpy change associated with their peptides binding to small unilamellar DMPC vesicles above the DMPC gel-to-liquid-crystalline phase transition temperature (from the absence of any apparent temperature dependence in van't Hoff plots of partition coefficient), approximately one-third of the free energy change of Z-D-Phe-L-Phe-Gly binding to 600 Å unilamellar DOPC vesicles is enthalpic, the remaining two-thirds being of entropic origin.

Regarding biological fusion, Z-D-Phe-L-Phe-Gly has been shown to cause a 50% reduction in the fusion rate above a concentration threshold of 100−200 μM (Richardson *et al.*, 1980). Similarly, in vesicle systems, Kelsey and co-workers found that 100 μM Z-D-Phe-L-Phe-Gly caused a 50% reduction in the fusion rate of 400 μM *N*-methyldioleoylphospatidylethanolamine (DOPE-Me) vesicles (Kelsey *et al.*, 1990, 1991). At pH 9.5, Kelsey *et al.* (1991) found that 35% of the peptide was bound to 4000 Å unilamellar DOPE-Me vesicles at these respective concentrations of peptide and lipid. Assuming a bilayer thickness of 60 Å (Gruner *et al.*, 1988), that only the outer monolayer of the vesicles is available for peptide interaction, and that the same lipid/peptide interaction stoichiometry as determined for 600 Å unilamellar DOPC vesicles holds in the case of DOPE-Me also (i.e., $n = 5.56$), then a value of log K for the Z-D-Phe-L-Phe-Gly/DOPE-Me binding (in terms of per mole of lipid bound) can be calculated to be 4.675. This corresponds to a free energy change for peptide binding to DOPE-Me vesicles of −36.9 kcal/mol of Z-D-Phe-L-Phe-Gly at 37 °C. This is nearly twice as favorable a binding free energy change as that for Z-D-Phe-L-Phe-Gly interaction with 600 Å DOPC vesicles and corresponds to over a 100-fold greater affinity of DOPE-Me over DOPC for binding sites on the peptide (i.e., a log K of 4.675 versus 2.430 at 37 °C). The interaction of Z-D-Phe-L-Phe-Gly with 4000 Å DOPE-Me vesicles is clearly much more heavily favored than its interaction with 600 Å DOPC vesicles under the respective conditions of these experiments. This could be interpreted to add support for the hypothesis of Yeagle *et al.* (1992) stating that Z-D-Phe-L-Phe-Gly stabilizes, and thus interacts more strongly with, lipid assemblies with a large, positive, radius of curvature. Alternatively, it could reflect a physical tendency for Z-D-Phe-L-Phe-Gly to hydrogen bond more strongly with DOPE-Me than DOPC since DOPE-Me can act as both a hydrogen bond donor and acceptor. Our data cannot distinguish between these two possibilities without adding a series of peptide binding measurements as a function of DOPC vesicle size.

Finally, an interesting thermodynamic property that emerges regarding Z-D-Phe-L-Phe-Gly binding to DOPC vesicles is the apparent temperature dependence of the heat capacity change of binding, ΔC_{p1}. Since binding enthalpy is the quantity measured using calorimetry, temperature changes in the binding heat capacity can be directly observed. Resolution of such an effect could probably not be expected from application of any other experimental technique other than calorimetry. One possible mechanistic interpretation of the temperature-dependent heat capacity change of binding can be developed as follows. Association of a peptide possessing exposed hydrophobic surface area into a lipid bilayer provides a mechanism for sequestration of hydrophobic surface area from the aqueous environment to a receptive hydrophobic environment (i.e., the hydrocarbon bilayer interior in the vicinity of the bilayer−water interface). Removing hydrophobic surface area from aqueous contact and placing it in the more hydrophobic environment of the lipid bilayer is expected to have an associated negative heat capacity change, as seen experimentally. The decreasing magnitude of this negative heat capacity change of binding as temperature is elevated is consistent with peptide partitioning into a less hydrophobic bilayer environment at higher temperatures. Increasing dynamic fluctuations in the structure of DOPC vesicles at the bilayer−water interface as temperature is increased would tend to facilitate greater probability of water penetration and residence in this interfacial region. As a consequence, the hydrophobicity experienced by vesicle-bound Z-D-Phe-L-Phe-Gly will de-

crease as temperature is raised, thus yielding a less negative binding heat capacity change.

CONCLUSION

Variable temperature isothermal titration calorimetry was used to thermodynamically characterize the weak-binding association of Z-D-Phe-L-Phe-Gly and DOPC bilayer membranes. A global strategy for simultaneously analyzing complete sets of temperature dependent calorimetric titration profiles permitted quantitative determination of binding stoichiometry, affinity, enthalpy change, and heat capacity change as well as the temperature dependence of the heat capacity change of binding. Results obtained for the interaction of Z-D-Phe-L-Phe-Gly with small unilamellar vesicles of DOPC, as summarized in Tables 1 and 3, attest to the general utility of calorimetric titrations for elucidation of peptide–lipid binding thermodynamics.

Comparison with results of binding studies reported for other peptide and lipid systems was discussed but is difficult due to the number of variable aspects inherent to various experimental protocols. Differing lipid compositions, vesicle sizes, peptide compositions, and experimental methods employed all tend to confound efforts for reliable direct comparisons of quantitative results regarding peptide–lipid binding affinities. In any case, the data in this paper provide circumstantial support for the Z-D-Phe-L-Phe-Gly/phospholipid interaction model proposed by Yeagle and co-workers (Yeagle *et al.*, 1992). In particular, the number of lipid molecules interacting with Z-D-Phe-L-Phe-Gly that was determined from the titration is in good agreement with their simple steric model of the peptide–lipid interaction. Further experiments, however, are required to investigate whether phospholipid curvature affects the binding affinity of Z-D-Phe-L-Phe-Gly as predicted by the Yeagle et al. model. These future studies are expected to further enhance our understanding of the relationships between peptide structure, thermodynamics of binding, and fusion inhibition.

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