

# Isothermal Titration Calorimetry Studies of the Binding of the Antimicrobial Peptide Gramicidin S to Phospholipid Bilayer Membranes<sup>†</sup>

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**ABSTRACT:** The binding of the amphiphilic, positively charged, cyclic  $\beta$ -sheet antimicrobial decapeptide gramicidin S (GS) to various lipid bilayer model membrane systems was studied by isothermal titration calorimetry. Large unilamellar vesicles composed of the zwitterionic phospholipid 1-palmitoyl-2-oleoylphosphatidylcholine or the anionic phospholipid 1-palmitoyl-2-oleoylphosphatidylglycerol, or a binary mixture of the two, with or without cholesterol, were used to mimic the lipid compositions of the outer monolayers of the lipid bilayers of mammalian and bacterial membranes, respectively. Dynamic light scattering results suggest the absence of major alterations in vesicle size or appreciable vesicle fusion upon the binding of GS to the lipid vesicles under our experimental conditions. The binding isotherms can be reasonably well described by a one-site binding model. GS is found to bind with higher affinity to anionic phosphatidylglycerol than to zwitterionic phosphatidylcholine vesicles, indicating that electrostatic interactions in the former system facilitate peptide binding. However, the presence of cholesterol reduced binding only slightly, indicating that the binding of GS is not highly sensitive to the order of the phospholipid bilayer system. Similarly, the measured positive endothermic binding enthalpy ( $\Delta H$ ) varies only modestly (2.6 to 4.4 kcal/mol), and the negative free energy of binding ( $\Delta G$ ) also remains relatively constant (−10.9 to −12.1 kcal/mol). The relatively large but invariant positive binding entropy, reflected in relatively large  $T\Delta S$  values (13.4 to 16.4 kcal/mol), indicates that GS binding to phospholipid bilayers is primarily entropy driven. Finally, the relative binding affinities of GS for various phospholipid vesicles correlate relatively well with the relative lipid specificity for GS interactions with bacterial and erythrocyte membranes observed *in vivo*.

The increasing number of infectious bacteria which are becoming resistant to conventional small molecule antibiotics represent an increasingly serious public health problem (see refs 1 and 2). Multidrug-resistant strains of an increasing number of bacteria now pose serious medical problems, particularly in hospitals and nursing homes. Moreover, the number of new antibiotics being developed by pharmaceutical firms, particularly those with novel mechanisms of action, is limited. These developments have prompted some researchers to raise the specter of a possible “post-antibiotic era”, where seemingly minor infections could turn serious and even lethal due to a lack of effective drugs with which to treat them.

A promising approach to the bacterial drug resistance problem is to utilize naturally occurring antimicrobial peptides or their synthetic analogues to combat bacterial or fungal infections (see refs 3–5). Such peptides are produced

by microorganisms to inhibit the growth of competitors or by plants and animals as part of their innate immune systems, and many of them are active against bacteria and fungi that are resistant to conventional antibiotics. Antimicrobial peptides exist in a wide variety of structural motifs ( $\alpha$ -helical,  $\beta$ -sheet, linear, and cyclic), but almost all are cationic amphiphiles which can interact with the bacterial inner membrane, which appears to be their primary target (see refs 6 and 7). The mode of action of such peptides is thought to involve an initial electrostatic interaction between anionic phospholipids on the outer surface of the bacterial membrane and the cationic amino acid residues of the peptide, followed by partial penetration of the peptide into the lipid bilayer, thus allowing the hydrophobic amino acid residues of the peptide to contact the hydrocarbon chains of the phospholipids. These peptides are then proposed to kill bacteria by disrupting the structural integrity of the phospholipid bilayer which, by compromising their permeability to ions and small molecules, causes a collapse of the transmembrane electrochemical potential, leading quickly to cell de-energization and death.

An appealing feature of antimicrobial peptides as potential antibiotics is that their rapid mode of action makes the development of resistance to them difficult. These small molecules either generally pass readily through bacterial outer membranes or cell walls or actually disrupt their structure

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and barrier integrity, making it difficult to prevent their access to the inner membrane. Moreover, since they generally do not have to enter bacterial cells to kill them, active efflux pumps do not protect the bacterial target. Also, many antimicrobial peptides contain compact structures and D-amino acids, thus increasing their resistance to proteolysis or derivatization. Finally, sequestration of the peptide by nontarget cellular proteins or the mutation or overproduction of target proteins is also ineffective, because the target of these agents is the lipid bilayer of the bacterial membrane. Although in principle drug resistance could arise from alterations in the lipid composition of the bacterial membrane, this is also unlikely, as bacteria typically have a limited membrane lipid biosynthetic capability and significant alterations in the lipid composition and physical properties of the lipid bilayer will disrupt membrane function and thus reduce viability. Moreover, the fact that the suite of antimicrobial peptides produced by plants and animals have retained their effectiveness over evolutionary time, and that *bone fide* reports of true resistance to specific antimicrobial peptides are rare, provides hope that bacterial resistance will develop slowly, if at all, and that such resistance will not spread as rapidly or as widely through bacterial populations (3–7).

Gramicidin S (GS)<sup>1</sup> is a cationic cyclic decapeptide of primary structure cyclo-(Val-Orn-Leu-D-Phe-Pro)<sub>2</sub> secreted by the bacterium *Bacillus brevis* (8). GS is a potent antimicrobial agent, exhibiting high killing activity against a broad spectrum of both Gram-positive and Gram-negative bacteria and pathogenic fungi (see refs 9 and 10). Unfortunately, GS is rather nonspecific and exhibits appreciable hemolytic as well as antimicrobial activity, thus restricting its use as an antibiotic to topical applications. However, we have recently shown that by simultaneously altering both GS ring size and the enantiomeric conformation of key amino acid residues, strongly antimicrobial GS analogues with markedly reduced hemolytic activity can be obtained (11–14). It is thus possible that appropriate GS analogues can be developed for use as potent oral or injectable broad-spectrum antibiotics for the treatment of infections caused by bacteria resistant to conventional antibiotics (see ref 5).

The three-dimensional structure of the GS molecule has been extensively studied by a wide range of physical techniques (see refs 9 and 10) and is illustrated in Figure 1. In this minimum energy conformation, the two Val-Orn-Leu sequences form an antiparallel  $\beta$ -sheet terminated by a Type II'  $\beta$ -turn formed by the two D-Phe-Pro sequences. This rather rigid structure is stabilized by four hydrogen bonds between the amide protons and carbonyl groups of the two Leu and two Val residues. The GS molecule is amphiphilic, having the two somewhat polar and charged Orn side chains and

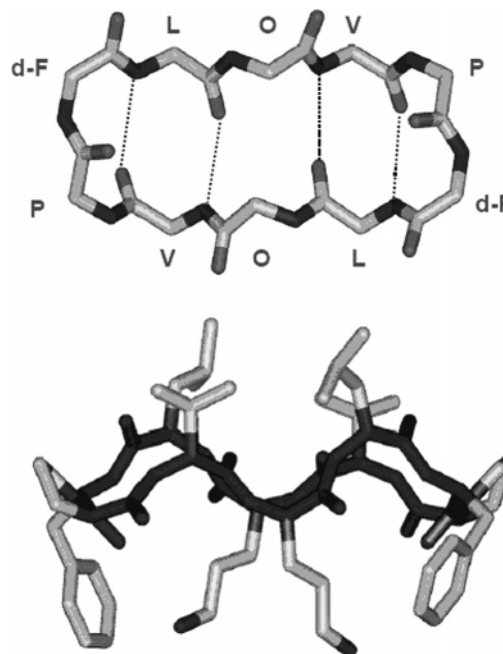


FIGURE 1: Structure and conformation of GS. The top panel is a view of the GS molecule perpendicular to the plane of the ring, illustrating the peptide backbone structure and the positions of the hydrogen bonds in the antiparallel L-sheet region. The bottom panel is a view of the GS molecule in the plane of the ring, indicating the disposition in space of the hydrophobic Val and Leu residues (top) and the basic Orn residues (bottom) relative to the peptide ring.

the two D-Phe rings projecting from one side of the molecule and the four hydrophobic Val and Leu side chains projecting from the other. This characteristic conformation of the GS molecule is maintained in water, in protic and aprotic organic solvents of widely varying polarity, and in detergent micelles and phospholipid bilayers, even at high temperatures and in the presence of agents which usually denature peptides and proteins. The conformational stability of GS can be a distinct advantage in certain biophysical studies, as illustrated below.

Considerable evidence exists that the major target of GS is the lipid bilayer of bacterial or erythrocyte membranes and that this peptide kills bacteria by permeabilizing and destabilizing their inner membranes (see refs 2, 3, 15, and 16). To determine the molecular mechanism of action of GS, we have recently carried out a number of biophysical studies of its interaction with a variety of lipid bilayer model membranes (see refs 15 and 16). In particular, we have shown by DSC that GS more strongly perturbs the thermotropic phase behavior of anionic rather than of zwitterionic phospholipid bilayers and of more fluid than of less fluid membranes (17) and by densitometry and sound velocity studies that GS incorporation decreases the density and volume compressibility of the host phospholipid bilayer by increasing the conformational disorder and motional freedom of the phospholipid hydrocarbon chains (18). Our <sup>31</sup>P-NMR (19) and X-ray diffraction (20) studies indicate that GS at low concentrations causes the thinning of phospholipid bilayers and can induce the formation of inverted nonlamellar cubic phases in phospholipid dispersions at higher concentrations. Our FTIR studies show that GS is located in the polar–apolar interfacial region of phospholipid bilayers and that it penetrates more deeply into anionic and more fluid bilayers (21). As well, several physical techniques indicate that the

<sup>1</sup> Abbreviations: GS, gramicidin S, cyclo[VOLdFPVOLdFP] (the amino acid immediately after the *d* is the D-enantiomer); GS14dK4, cyclo[VKLdKVdYPLKVdYPL]; PC, phosphatidylcholine; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(glycerol)] (sodium salt); ITC, isothermal titration calorimetry; LUV, large unilamellar vesicle;  $\Delta H$ , total binding enthalpy;  $\Delta G$ , free energy of binding;  $\Delta S$ , entropy of binding;  $h_i$ , heat of reaction per injection;  $h_{d,i}$ , heat of dilution;  $X_b^i$ , degree of binding, fraction of bound peptide per mole of total lipid;  $c_f^i$ , concentration of the peptide remaining free in solution after the *i*th injection;  $K_c^i$ , concentration-dependent binding constant;  $K_c$ , equilibrium binding constant.

presence of cholesterol attenuates all of the above effects of GS on phospholipid bilayers, at least in part by reducing the penetration of the peptide into these model membranes (21, 22). Finally, our solid-state  $^{19}\text{F}$ -NMR study of a  $^{19}\text{F}$ -labeled GS analogue suggests that the GS molecule is aligned with its cyclic  $\beta$ -sheet lying flat in the plane of the bilayer, with its polar and positively charged Orn residues projecting toward the bilayer surface, where they can interact with negatively charged phosphate headgroups of the phospholipid molecules, while the four hydrophobic Val and Leu residues project toward the bilayer center, where they interact with the phospholipid hydrocarbon chains (23).

Although these and other studies provide considerable insight into the interactions of GS with a variety of lipid model membranes, there remains a clear need to characterize the actual binding of GS to various lipid bilayer systems in a rigorous and quantitative manner. In this study, we have utilized ITC for this purpose, as this technique provides a comprehensive thermodynamic description of the entire binding process (24–26). In particular, an accurate determination of the association constant,  $K_C$ , permits a quantitative determination of the peptide water/phospholipid bilayer partition coefficients and relative degree of phospholipid binding specificity. Moreover, a complete thermodynamic characterization of the binding process can provide important information about the energetics and mechanism of peptide binding to lipid bilayers. In this study, we characterize the binding of GS to zwitterionic POPC and anionic POPG lipid bilayer model membranes in the presence and absence of cholesterol. PCs are the major phospholipids of the outer monolayer of the lipid bilayer of animal cell membranes, while PGs are the major anionic phospholipid of bacterial cell membranes, and cholesterol is present only in animal cell membranes. This study should thus provide insight into the relative strength of GS binding to bilayers mimicking the lipid compositions of the membranes of erythrocytes and bacteria, respectively.

## MATERIALS AND METHODS

**Materials.** Phospholipids and cholesterol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and used without further purification. A linear version of GS was synthesized by solid-phase peptide synthesis using *tert*-butyloxycarbonyl chemistry. The linear peptide was then N- to C-terminally (Pro at the C-terminus) cyclized in solution to produce GS, after which it was purified by RP-HPLC, and the final purity was determined by mass spectrometry, all as previously described (27). The concentration of GS in aqueous stock solutions was determined by amino acid analysis with a range of error of  $\pm 5\%$ . All experiments, unless otherwise stated, were conducted in a buffer solution containing 50 mM Tris, 150 mM NaCl, and 1 mM  $\text{NaN}_3$  (pH 7.4).

**Preparation of LUVs.** The single-component lipid vesicles were prepared as follows. In a typical experiment, specified amounts of lipid ( $\sim 60$  mg) were first dried under reduced pressure (vacuum) overnight. The lipid was then hydrated with a definite amount of buffer ( $\sim 2$  ml), and the dispersion thus formed was subjected to vortex mixing at temperatures well above the gel/liquid-crystalline phase transition temperature of the phospholipid. The multilamellar vesicles thus

obtained were then frozen and thawed several times. These vesicles were then extruded through a small volume extrusion apparatus (Avestin Inc., Ottawa, ON) equipped with a polycarbonate membrane filter (19 mm diameter, 200 nm pore diameter)  $\sim 25$  times, to produce LUVs with a vesicle size of  $\sim 200$  nm. The same procedure was followed for two-component vesicles, except that appropriate quantities of each component (lipid or cholesterol) were first codissolved in chloroform and thoroughly mixed prior to vesicle preparation.

Phospholipid concentrations were determined by gas chromatographic analysis of fatty acid esters produced by transesterification with acidic methanol, using an appropriate internal standard ( $\pm 5\%$  error), all as described previously (28).

**High-Sensitivity Isothermal Titration Calorimetry.** The heat flow resulting from the binding of the peptide to lipid vesicles was measured using a high-sensitivity VP-ITC instrument (Microcal LLC, Northampton, MA) with a reaction cell volume of 1.4448 ml as described previously (29). Prior to use, solutions were degassed under vacuum (140 mbar, 8 min) to eliminate air bubbles. The data were acquired by computer software developed by MicroCal LLC. Titration calorimetric experiments were performed as follows. The peptide solution (10–60  $\mu\text{M}$ ) was placed in the calorimeter cell, and the lipid vesicles (1.0–38 mM) were injected via the titration syringe in aliquots of 3–10  $\mu\text{L}$ . Each injection produced a heat of reaction,  $h_i$ , which was determined by integration of the heat flow tracings. This mode is capable of providing both the binding isotherm and the total binding enthalpy.

The heat of dilution,  $h_{d,i}$ , was determined in control experiments by injecting the corresponding vesicle dispersion into the buffer solution. The heats of dilution were subtracted from the heats determined in the corresponding peptide–lipid binding experiments. Thus, the quantitative evaluation of the experimental data was based on the relationship  $\Delta h_i = h_i - h_{d,i}$ . The overall binding enthalpy and the binding isotherm were determined from these peptide–lipid binding experiments using standard procedures (24–26). In particular, the reduction in peptide concentration due to the increase in volume by vesicle injection was taken into account by incorporating a dilution factor in the calculations (26). In this study, since we do not have any direct experimental evidence to establish the transmembrane distribution of the peptide molecules, we used the total lipid concentration in the calculation of the degree of binding and the determination of the thermodynamic binding parameters.

**Dynamic Light Scattering.** The average hydrodynamic diameter of pure and peptide-bound LUVs was measured by a Brookhaven BI-90 particle analyzer (Brookhaven Instruments, Holtsville, NY) using disposable square cells. The solutions were subjected to scattering by a monochromatic light (10 mW He–Ne laser, wavelength of 632.4 nm), and the scattered light intensity was measured at a scattering angle of  $90^\circ$ .

## RESULTS

**Size of Pure and Peptide-Bound LUVs.** It has been suggested previously that GS may induce phospholipid vesicle fusion or even solubilize phospholipid, at least at higher peptide concentrations (see refs 9, 10, and 15). To



Table 1: Hydrodynamic Diameter of Pure and Peptide-Bound LUVs

model membrane	hydrodynamic diameter ( <i>d</i> ) (nm)	
	in buffer	in GS solution
POPC	166	175
POPG	178	188
POPC/POPG (3:1)	179	187
POPC/cholesterol (6:4)	210	228
POPG/cholesterol (6:4)	179	192

check the integrity of the LUVs exposed to GS, dynamic light scattering measurements were performed on both control vesicles (LUVs titrated into buffer solution without peptide) and the sample vesicles (LUVs titrated into the peptide-containing buffer). The data are presented in Table 1. Although the hydrodynamic diameters (*d*) of the peptide-exposed lipid vesicles are always slightly greater than those of pure lipid vesicles, these results indicate that the various LUVs studied here remain largely intact and do not undergo extensive fusion in the presence of GS under the experimental conditions employed in our ITC measurements.

**Binding of GS to Various Phospholipid LUVs.** ITC experiments, in which LUVs composed of the various phospholipid or phospholipid/cholesterol compositions studied were titrated into GS, are shown in Figure 2. In particular, Figure 2A illustrates an ITC experiment in which 5  $\mu$ L aliquots of POPC LUVs (10 mM) were repeatedly injected into the reaction cell containing GS (25  $\mu$ M). As is evident from the titration profile, the endothermic heat flow decreases with the increase in the number of POPC injections as the free peptide concentration in the vessel progressively decreases. The endothermic binding reaction essentially ceases after six injections, when all of the peptide molecules present in the reaction vessel are bound to the lipid vesicles. Comparison of the heat released by the first injection (+31  $\mu$ cal) to the cumulative heat released (+97  $\mu$ cal) suggests that approximately one-third of the total amount of the antimicrobial peptide in the reaction cell was bound in a single injection of the zwitterionic POPC lipid vesicles. The total binding enthalpy ( $\Delta H$ ) is determined to be 2.7 kcal/mol.

The two important parameters which constitute the binding isotherm, namely, the degree of binding,  $X_b^i$ , i.e., the fraction of bound peptide per mole of total lipid, and the corresponding free peptide concentration,  $c_f^i$ , determined from the titration experiments, are shown in Figure 3. The data points represent individual titration steps. Under these conditions, there should be an equilibrium between the peptide in the vesicle-bound state and the peptide in the aqueous solution (free peptide) (24–26). Therefore,  $X_b^i$  should have a functional dependence on the  $c_f^i$ , i.e.,  $X_b^i = f(c_f^i)$ . For practical purposes, one can obtain the concentration-dependent binding constant,  $K_c^i$ , corresponding to a particular equilibrium peptide concentration ( $c_f^i$ ), directly from this binding isotherm by  $K_c^i = X_b^i/c_f^i$ . For this system,  $K_c^i$  varies considerably from  $1.5 \times 10^5$  M<sup>-1</sup> when  $c_f^i = 0.8$   $\mu$ M to  $1.4 \times 10^4$  M<sup>-1</sup> when  $c_f^i = 16.9$   $\mu$ M. Attempts have been also made to quantify the functional dependency,  $X_b^i = f(c_f^i)$ , using a one-site binding model [ $X_b^i = (B_{\max} K_c c_f^i)/(1 + K_c c_f^i)$ ]. In this one-site binding model equation,  $B_{\max}$  is the maximal binding capacity (same unit as that of  $X_b^i$ , i.e.,

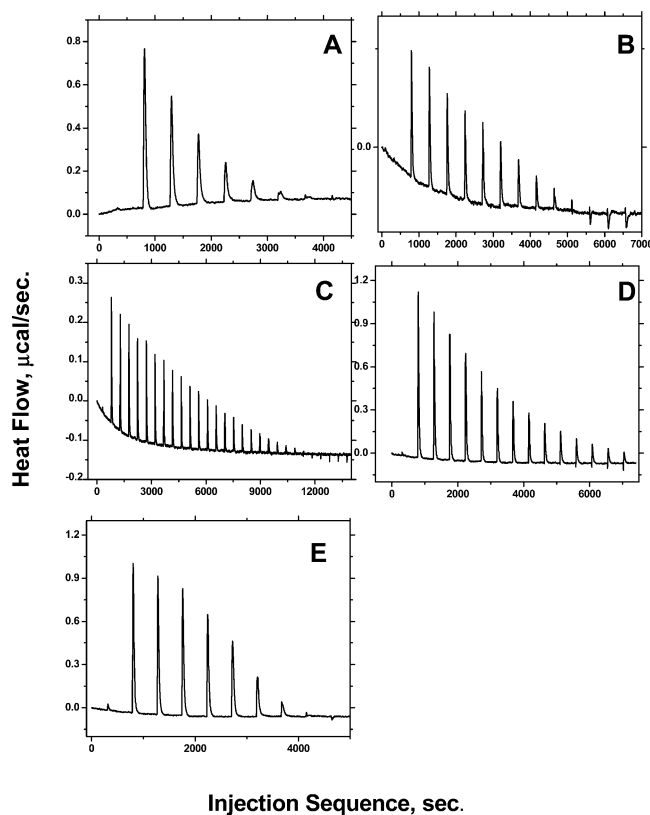


FIGURE 2: ITC experiments conducted at 25 °C, where phospholipid LUVs (1–10 mM) were repeatedly injected into the reaction cell (1.4448 mL) containing GS (10–50  $\mu$ M). Each peak refers to the injection. Each injection produced an endothermic heat of reaction which decreased in magnitude with subsequent injections. All of the peptide molecules present in the reaction vessel appear to be bound to the lipid vesicles after several injections: (A) titration of 5  $\mu$ L aliquots of 10 mM POPC LUVs into 25  $\mu$ M GS, (B) titration of 3  $\mu$ L aliquots of 1.0 mM POPG LUVs into 10  $\mu$ M GS, (C) titration of 4  $\mu$ L aliquots of 2.5 mM POPC/POPG (3:1) LUVs into 25  $\mu$ M GS, (D) titration of 5  $\mu$ L aliquots of 10 mM POPC/cholesterol (6:4) LUVs into 50  $\mu$ M GS, and (E) titration of 4  $\mu$ L aliquots of 2.5 mM POPG/cholesterol (6:4) LUVs into 25  $\mu$ M GS.

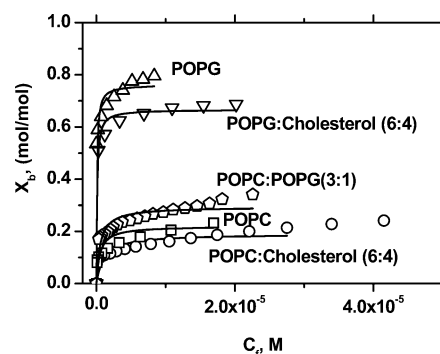


FIGURE 3: Binding isotherms for binding of GS to various LUVs derived from the ITC measurements at 25 °C. The degree of binding ( $X_b^i$ ) is plotted as a function of free peptide concentration ( $c_f^i$ ). Each data point represents an individual titration step. The solid lines represent theoretical fits according to the one-site binding model. The binding capacity ( $B_{\max}$ ) and binding constants ( $K_c$ ), obtained from the theoretical fit, are summarized in Table 2.

moles per mole) and  $K_c$  is the equilibrium binding constant (inverse units of  $c_f^i$ , i.e., M<sup>-1</sup>). The one-site binding model describes the binding curve fairly well (solid lines in Figure 3) and yields a  $K_c$  of  $2.0 \times 10^6$  M<sup>-1</sup> and a  $B_{\max}$  value of 0.22. Several other empirical models have also been used to

Table 2: Thermodynamic Parameters for the Binding of GS to Various LUVs

system	$\Delta H$ (kcal/mol)	$K_c^i$ <sup>a</sup> (M <sup>-1</sup> )	$B_{\max}$ (mol/mol)	$K_c$ (M <sup>-1</sup> )	$\Delta G$ (kcal/mol)	$T\Delta S$ (kcal/mol)	$\Delta S$ (cal mol <sup>-1</sup> K <sup>-1</sup> )
POPC	2.7	$1.46 \times 10^5$ (0.8 $\mu$ M) $1.37 \times 10^4$ (16.9 $\mu$ M)	0.22	$2.0 \times 10^6$	-11.0	13.7	45.9
POPG	2.6	$9.3 \times 10^5$ (at 0.7 $\mu$ M) $9.6 \times 10^4$ (at 8.3 $\mu$ M)	0.76	$1.3 \times 10^7$	-12.1	14.6	49.2
POPC/POPG (3:1)	2.6	$2.4 \times 10^5$ (0.8 $\mu$ M) $1.5 \times 10^4$ (22.6 $\mu$ M)	0.29	$1.9 \times 10^6$	-10.9	13.4	45.3
POPC/cholesterol (6:4)	3.1	$2.6 \times 10^4$ (11.0 $\mu$ M) $2.7 \times 10^4$ (21.0 $\mu$ M)	0.19	$9.1 \times 10^5$	-10.5	13.7	45.8
POPG/cholesterol (6:4)	4.4	$4.8 \times 10^5$ (1.2 $\mu$ M) $3.4 \times 10^4$ (20.2 $\mu$ M)	0.67	$1.0 \times 10^7$	-11.9	16.4	54.9

<sup>a</sup> Concentration-dependent binding constant. The corresponding equilibrium peptide concentration ( $c_p^i$ ) is shown in parentheses.

describe the binding (or partitioning) of peptide to (or into) lipid bilayer membranes (30). Considering the diversity of the model membrane systems studied (see below), we adopted an empirical model, i.e., the one-site binding model, which can well describe the binding isotherms generated in this study. The main purpose of using this empirical description is to obtain the binding capacity ( $B_{\max}$ ) and the binding constant ( $K_c$ ) so that one can determine all the relevant thermodynamic parameters ( $\Delta G$  and  $T\Delta S$ ) and compare them among the various lipid model membrane systems utilized in this study.

The binding between the POPC membrane and GS can thus be characterized thermodynamically by its  $K_c$  and  $\Delta H$ . The corresponding free energy of binding,  $\Delta G$ , can be calculated using the standard relation  $\Delta G = -RT \ln(55.5K_c)$ , where the factor 55.5 is the molar concentration of water in dilute solution that corrects for the cratic contribution to the free energy (31). The  $K_c$  value obtained for GS binding to POPC LUVs is  $2.0 \times 10^6$  M<sup>-1</sup>, and the  $\Delta G$ , i.e., the free energy change that accompanies the binding of the peptide to the lipid membrane, is determined to be -11.0 kcal/mol. The value of  $\Delta G$  provides a quantitative measurement of the strength of the binding such that the more negative the  $\Delta G$ , the stronger the binding. Finally, from these thermodynamic parameters, the binding reaction entropy ( $\Delta S$ ) can be calculated using the equation  $\Delta S = (\Delta H - \Delta G)/T$ . The  $\Delta S$  in this case is found to be 45.9 cal mol<sup>-1</sup> K<sup>-1</sup>. Thus, the binding of GS to the POPC membrane is an entropy-driven process with a positive  $T\Delta S$  of 13.7 kcal/mol at 25 °C, but counteracted by a positive  $\Delta H$  of 2.7 kcal/mol (see Table 2 for compiled thermodynamic data). These thermodynamic parameters are in agreement with the classical understanding of the partitioning of a nonpolar molecule from a water phase into a nonpolar phase, which is also accompanied by an increase in the entropy of the system (32).

Figure 2B shows a titration experiment in which 4  $\mu$ l aliquots of 2.5 mM POPG LUVs are introduced into an ITC cell containing a 25  $\mu$ M GS solution, and the resultant binding isotherm for the POPG/GS system ( $X_b^i$  vs  $c_p^i$ ) is shown in Figure 3. It is immediately evident from Figure 3 that the maximum amount of GS bound to the anionic POPG LUVs ( $B_{\max} = 0.76$ ) is substantially higher than that bound to the zwitterionic POPC LUVs ( $B_{\max} = 0.22$ ). Moreover, the strength of binding of GS to anionic POPG LUVs, as reflected in the  $K_c$  and  $\Delta G$  values ( $1.3 \times 10^7$  M<sup>-1</sup> and -12.1 kcal/mol, respectively), is greater than for the binding of this peptide to zwitterionic POPC LUVs, although binding of GS

to the POPG vesicles is accompanied by a similar positive enthalpy ( $\Delta H = 2.6$  kcal/mol) but by somewhat larger  $\Delta S$  and  $T\Delta S$  values (49.2 cal mol<sup>-1</sup> K<sup>-1</sup> and 14.6 kcal/mol, respectively). Thus, the presence of a negative surface charge on the POPG bilayer surface appears to increase both the maximum capacity and the strength of GS binding, but the binding process is again entropically driven. As might be expected, the strength and energetics of GS binding to POPC/POPG (3:1) LUVs are generally more similar to those of the zwitterionic POPC than for the anionic system, since POPC is the major component of these two-component LUVs (see Figures 2C and 3 and Table 2).

The titrations and binding isotherms for the binding of GS to both zwitterionic POPC and anionic POPG LUVs containing 40 mol % cholesterol are also shown in panels D and E of Figure 2 and Figure 3, and the derived thermodynamic parameters for peptide binding to these systems are compiled in Table 2. The addition of cholesterol to the zwitterionic POPC LUVs reduces the  $K_c$  and  $\Delta G$  values slightly, primarily by increasing the magnitude of the positive enthalpy of binding, while having no significant effect on the  $\Delta S$  and  $T\Delta S$  values and only slightly reducing the  $B_{\max}$  values. In contrast, the addition of cholesterol to the anionic POPG vesicles significantly decreases the  $K_c$  and perhaps also  $\Delta G$  values for GS binding, primarily through moderate increases in the magnitude of the  $\Delta S$  and  $T\Delta S$  values for peptide binding and despite the increased positive enthalpy of binding. Also, a modest reduction in the  $B_{\max}$  value also occurs upon addition of 40 mol % cholesterol to anionic POPG LUVs. In both POPC/cholesterol and POPG/cholesterol vesicle systems, the peptide binding process is again entropically driven, as in the phospholipid vesicle systems not containing cholesterol.

## DISCUSSION

The ITC results obtained here are generally compatible with those obtained in our previous biophysical studies of the interaction of GS with zwitterionic and anionic phospholipid model membranes containing or not containing cholesterol (see refs 15 and 16). For example, our DSC studies indicate that GS incorporation produces considerably greater decreases in the temperature, enthalpy, and cooperativity of the gel/liquid-crystalline phase transition of anionic DMPC than of zwitterionic DMPC membranes (17) and that the presence of cholesterol reduces the effect of GS on the thermotropic phase behavior of DMPC (22). Moreover, our previous FTIR spectroscopic studies demonstrate that GS binds to a greater degree and/or penetrates more deeply into

bilayers composed of anionic phospholipids such as DMPG than of zwitterionic phospholipids such as DMPC (19). Also, subsequent FTIR studies revealed that the degree of GS binding to and/or the depth of penetration into zwitterionic POPC bilayers is reduced somewhat in the presence of cholesterol (22). Our previous  $^{31}\text{P}$ -NMR studies have also shown that GS at higher concentrations can induce an inverted cubic phase more readily in anionic DMPG than in zwitterionic DMPC vesicles (19), and that the presence of cholesterol reduces the likelihood of phospholipids generally forming such a nonlamellar phase at high temperatures (22). Finally, the incorporation of cholesterol into POPC LUVs reduces GS-induced dye leakage by approximately half (22). It should be pointed out that all of the above studies, with the exception of the dye leakage experiments, were performed with phospholipid bilayer membranes into which GS was reconstituted by codissolution with various phospholipids prior to hydration and vesicle formation. Therefore, GS was uniformly distributed on both sides of the phospholipid bilayer from the outset rather than being added to preformed LUVs, as in the case of the ITC experiments described here. Moreover, these previous biophysical studies may also have been measuring the intrinsic effects of GS on the organization and dynamics of the host phospholipid bilayer, as well as the amount of GS actually present in the bilayer during these experiments. Nevertheless, the results of these earlier studies are certainly broadly consistent with those of this ITC investigation, in that in all instances GS appeared to interact more strongly with anionic PG than with zwitterionic PC vesicles, and GS interactions with PC bilayers were always attenuated but not abolished by the presence of cholesterol.

It is interesting to compare the results of our recent ITC study of the binding of GS14dK<sub>4</sub> (29), a diastereomeric lysine ring-size analogue of GS, to those obtained in this ITC study of GS binding to phospholipid vesicles of comparable compositions. In general, the strength of binding of GS14dK<sub>4</sub> is much more significantly influenced by the phospholipid and cholesterol composition of the LUVs, in particular by phospholipid surface charge density and cholesterol-induced ordering of the phospholipid bilayer, than is the binding of GS. For example, the binding of GS14dK<sub>4</sub> to zwitterionic POPC LUVs is much weaker than that of GS, and GS14dK<sub>4</sub> does not exhibit detectable binding to POPC/cholesterol (6:4) LUVs; whereas binding of GS to POPC LUVs is only weakened slightly by the presence of cholesterol. In contrast, GS14dK<sub>4</sub> binds only slightly less tightly to anionic POPG LUVs than does GS, although the binding of GS14dK<sub>4</sub> to POPG/cholesterol (6:4) LUVs is markedly reduced, while the binding of GS to POPG LUVs is affected little by the presence of cholesterol. Moreover, the binding of GS14dK<sub>4</sub> to all phospholipid vesicles that have been studied is accompanied by much higher positive  $\Delta H$  values, but also much larger  $\Delta S$  and  $T\Delta S$  values, than is GS binding such that the  $K_c$  and  $\Delta G$  values for binding for GS14dK<sub>4</sub> vary over a much larger range in different lipid vesicles than is the case for GS. As well, the  $B_{\text{max}}$  values of GS14dK<sub>4</sub> ( $\sim 0.10$ – $0.20$  mol/mol) are smaller than those observed for GS ( $\sim 0.2$ – $0.8$ ) in comparable systems, particularly in the case of anionic LUVs. These ITC results correlate well with our previous FTIR spectroscopic studies of the interactions of GS14dK<sub>4</sub> (33) and GS (21) with phospholipid vesicles, which indicate that GS14dK<sub>4</sub> binds less extensively to PG

and particularly to PC bilayers than does GS, and that the presence of cholesterol results in the complete exclusion of GS14dK<sub>4</sub> from PC bilayers but only the partial exclusion of GS.

Many of the results mentioned above can be rationalized by a consideration of the relative sizes and charge densities of the GS14dK<sub>4</sub> and GS molecules. Since GS14dK<sub>4</sub> is considerably larger than GS, one would expect that it would generally be more energetically costly to insert this peptide molecule into phospholipid vesicles, particularly those not bearing a negative surface charge and those exhibiting cholesterol-induced decreases in fluidity and increases in order, as is indeed observed. This suggestion is supported by the relatively much higher positive  $\Delta H$  values associated with the binding of GS14dK<sub>4</sub> to phospholipid vesicles, particularly those containing cholesterol. In contrast, the larger size of the GS14dK<sub>4</sub> molecule, and the fact that the somewhat polar and charged D-Lys residue projects toward the hydrocarbon chains of the phospholipid molecules rather than toward the polar headgroups, would tend to more strongly disorder the host phospholipid bilayer, again consistent with the higher  $\Delta S$  and  $T\Delta S$  values observed for the binding of GS14dK<sub>4</sub> compared to those for the binding of GS, particularly in cholesterol-containing phospholipid vesicles. On the other hand, the GS14dK<sub>4</sub> molecule contains three positively charged Lys residues positioned to interact with the phospholipid polar headgroups, whereas GS contains only two positively charged Orn residues so positioned. Thus electrostatic binding of the larger GS14dK<sub>4</sub> molecule to anionic phospholipids might not be as limited when compared with that of GS, as was the case with the zwitterionic phospholipids vesicles.

The binding data presented here for GS and previously for GS14dK<sub>4</sub> (29) correlate nicely with the biological activities of these two peptides. As mentioned previously, GS is a strong antimicrobial agent but is also highly hemolytic, suggesting that it binds relatively strongly to both bacterial membranes, which are enriched in anionic phospholipids such as PG and which do not contain cholesterol, and to erythrocyte membranes, the outer monolayer of which is enriched in zwitterionic lipids such as PC and which contain large amounts of cholesterol. The fact that this ITC study indicates that GS binding is relatively insensitive to the composition and physical properties of phospholipid vesicles is consistent with the relative lack of GS specificity observed *in vivo*. In contrast, GS14dK<sub>4</sub> retains the relatively strong antimicrobial potency of GS but is markedly less hemolytic. This relatively high specificity for interaction with bacterial membranes in comparison to erythrocyte membranes is fully consistent with our previous ITC results, which demonstrated that GS14dK<sub>4</sub> binds at least as tightly to anionic lipid vesicles as GS but much less strongly to zwitterionic lipid vesicles, and that the presence of cholesterol markedly decreases the level of GS14dK<sub>4</sub> binding to both anionic and zwitterionic phospholipid bilayer systems. Thus, the relatively low membrane specificity of GS and the relatively high membrane specificity of GS14dK<sub>4</sub> observed *in vivo* are consistent with their relative abilities to discriminate poorly and well, respectively, between bacterial and mammalian cell membranes based on their different lipid compositions and physical properties.



## REFERENCES

- Lohner, K., and Staudegger, E. (2001) Are we on the threshold of the post-antibiotics era? in *Development of Novel Antimicrobial Agents: Emerging Strategies* (Lohner, K., Ed.) pp 149–165, Horizon Scientific Press, Wymondham, U.K.
- Hall, R. M., and Collis, C. M. (2001) Origins and evolution of antibiotic and multiple antibiotic resistance in bacteria, in *Development of Novel Antimicrobial Agents: Emerging Strategies* (Lohner, K., Ed.) pp 1–15, Horizon Scientific Press, Wymondham, U.K.
- Grantz, T., and Lehrer, R. I. (2001) Antimicrobial peptides in innate immunity, in *Development of Novel Antimicrobial Agents: Emerging Strategies* (Lohner, K., Ed.) pp 139–147, Horizon Scientific Press, Wymondham, U.K.
- Zasloff, M. (2001) The commercial development of the antimicrobial peptide Pexiganan, in *Development of Novel Antimicrobial Agents: Emerging Strategies* (Lohner, K., Ed.) pp 261–270, Horizon Scientific Press, Wymondham, U.K.
- Lee, D. L., and Hodges, R. S. (2003) Structure–activity relationships of *de novo* designed cyclic antimicrobial peptides based on gramicidin S, *Biopolymers* 71, 28–48.
- Epand, R. M., and Vogel, H. J. (1999) Diversity of antimicrobial peptides and their mechanisms of action, *Biochim. Biophys. Acta* 1462, 11–28.
- Sitaram, N., and Nagaraj, R. (1999) Interaction of antimicrobial peptides with biological and model membranes: Structural and charge requirements for activity, *Biochim. Biophys. Acta* 1462, 29–54.
- Gause, G. G., and Brazhnikova, M. G. (1944) Gramicidin S and its use in the treatment of infected wounds, *Nature* 154, 703.
- Izumiya, N., Kato, T., Aoyaga, H., Waki, M., and Kondo, M. (1979) *Synthetic Aspects of Biologically Active Cyclic Peptides: Gramicidin S and Tyrocidines*, Halsted Press, New York.
- Waki, M., and Izumiya, N. (1990) Recent advances in the biotechnology of  $\beta$ -lactams and microbial bioactive peptides, in *Biochemistry of Peptide Antibiotics* (Kleinhaug, H., and van Dohren, H., Eds.) pp 205–240, Walter de Gruyter Co., Berlin.
- Kondejewski, L. H., Farmer, S. W., Wishart, D. S., Kay, C. M., Hancock, R. E. W., and Hodges, R. H. (1996) Modulation of structure and antibacterial and hemolytic activity by ring size in cyclic gramicidin S analogs, *J. Biol. Chem.* 271, 25261–25268.
- Kondejewski, L. H., Jelokhani-Niaraki, M., Farmer, S. W., Lix, B., Kay, C. M., Sykes, B. D., Hancock, R. E. W., and Hodges, R. S. (1999) Dissociation of antimicrobial and hemolytic activities in cyclic peptide diastereomers by systematic alterations in amphipathicity, *J. Biol. Chem.* 274, 13181–13192.
- Jelokhani-Niaraki, M., Kondejewski, L. H., Farmer, S. W., Hancock, R. E. W., Kay, C. M., and Hodges, R. S. (2000) Diastereoisomeric analogues of gramicidin S: Structure, biological activity, and interaction with lipid bilayers, *Biochem. J.* 349, 747–755.
- Kondejewski, L. H., Lee, D. L., Jelokhani-Niaraki, M., Farmer, S. W., Hancock, R. E., and Hodges, R. S. (2002) Optimization of microbial specificity in cyclic peptides by modulation of hydrophobicity within a defined structural framework, *J. Biol. Chem.* 277, 67–74.
- Prenner, E. J., Lewis, R. N. A. H., and McElhaney, R. N. (1999) The interaction of the antimicrobial peptide gramicidin S with lipid bilayer and biological membranes, *Biochim. Biophys. Acta* 1462, 201–221.
- Prenner, E. J., Lewis, R. N. A. H., and McElhaney, R. N. (2004) Biophysical studies of the interaction of the antimicrobial peptide gramicidin S with lipid bilayer model and biological membranes, *Phys. Can.* 60, 121–129.
- Prenner, E. J., Lewis, R. N. A. H., Kondejewski, L. H., Hodges, R. S., and McElhaney, R. N. (1999) Differential scanning calorimetric study of the effect of the antimicrobial peptide gramicidin S on the thermotropic phase behavior of phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol bilayer membranes, *Biochim. Biophys. Acta* 147, 211–223.
- Krivanek, R., Rybar, P., Prenner, E. J., McElhaney, R. N., and Hianik, T. (2001) Interaction of the antimicrobial peptide gramicidin S with dimyristoylphosphatidylcholine membranes: A densitometry and sound velocity study, *Biochim. Biophys. Acta* 1510, 452–463.
- Prenner, E. J., Lewis, R. N. A. H., Kondejewski, L. H., Hodges, R. S., and McElhaney, R. N. (1997) Nonlamellar phases induced by the interaction of gramicidin S with lipid bilayers. A possible relationship to membrane-disrupting activity, *Biochemistry* 36, 7906–7916.
- Staudegger, E., Prenner, E. J., Kriechbaum, M., Degovics, G., Lewis, R. N. A. H., McElhaney, R. N., and Lohner, K. (2000) X-ray studies on the interaction of gramicidin S with microbial lipid extracts: Evidence for cubic phase formation, *Biochim. Biophys. Acta* 1468, 213–230.
- Lewis, R. N. A. H., Prenner, E. J., Kondejewski, L. H., Flach, C. R., Mendelsohn, R., Hodges, R. S., and McElhaney, R. N. (1999) Fourier transform infrared spectroscopic studies of the interaction of the antimicrobial peptide gramicidin S with lipid micelles and with lipid monolayer and bilayer membranes, *Biochemistry* 38, 15193–15203.
- Prenner, E. J., Lewis, R. N. A. H., Jelokhani-Niaraki, M., Hodges, R. S., and McElhaney, R. N. (2001) Cholesterol attenuates the interaction of the antimicrobial peptide gramicidin S with phospholipid bilayer membranes, *Biochim. Biophys. Acta* 1510, 83–92.
- Salgado, J., Grage, S. L., Kondejewski, L. H., Hodges, R. S., McElhaney, R. N., and Ulrich, A. S. (2001) Membrane-bound structure and alignment of the antimicrobial  $\beta$ -sheet peptide gramicidin S derived from angular and distance constraints by solid-state  $^{19}\text{F}$  NMR, *J. Biomol. NMR* 21, 191–208.
- Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. (1989) Rapid measurement of binding constants and heats of binding using a new titration calorimeter, *Anal. Biochem.* 17, 131–137.
- Seelig, J. (1997) Titration calorimetry of lipid-peptide interactions, *Biochim. Biophys. Acta* 1331, 103–116.
- Wieprecht, T., and Seelig, J. (2002) Isothermal titration calorimetry for studying interactions between peptides and lipid membranes, *Curr. Top. Membr.* 52, 31–56.
- Kondejewski, L. H., Farmer, S. W., Wishart, D. S., Hancock, R. E. W., and Hodges, R. H. (1996) Gramicidin S is active against both Gram-positive and Gram-negative bacteria, *Int. J. Pept. Protein Res.* 47, 460–466.
- Lewis, R. N. A. H., and McElhaney, R. N. (1985) The thermotropic phase behavior of model membranes composed of phosphatidylcholines containing isobranched fatty acids. 1. Differential scanning calorimetric studies, *Biochemistry* 24, 2431–2439.
- Abraham, T., Lewis, R. N. A. H., Hodges, R. S., and McElhaney, R. N. (2005) Isothermal titration calorimetry studies of the binding of a rationally designed analog of the antimicrobial peptide gramicidin S to phospholipid bilayer membranes, *Biochemistry* 44, 2103–2112.
- White, S. H., Wimley, W. C., Ladokhin, A. S., and Hristova, K. (1998) Protein folding in membranes: Determining energetics of peptide-bilayer interactions, *Methods Enzymol.* 295, 62–87.
- Cantor, C. R., and Schimmel, P. R. (1980) *Biophysical Chemistry Part I: The Conformation of Biological Macromolecules*, Chapter 5, W. H. Freeman and Co., San Francisco.
- Tanford, C. (1980) *The Hydrophobic Effect*, John Wiley & Sons, Inc., New York.
- Lewis, R. N. A. H., Kiricsi, M., Prenner, E. J., Hodges, R. S., and McElhaney, R. N. (2003) Fourier transform infrared spectroscopic study of the interactions of a strongly antimicrobial but weakly hemolytic analog of gramicidin S with lipid micelles and lipid bilayer membranes, *Biochemistry* 42, 440–449.

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