Review

# The interaction of the antimicrobial peptide gramicidin S with lipid bilayer model and biological membranes

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## Abstract

Gramicidin S (GS) is a cyclic decapeptide of primary structure [cyclo-(Val-Orn-Leu-D-Phe-Pro)$_2$] secreted by *Bacillus brevis*. It is a powerful antimicrobial agent with potent cidal action on a wide variety of Gram-negative and Gram-positive bacteria as well as on several pathogenic fungi. Unfortunately, however, GS is rather non-specific in its actions and also exhibits a high hemolytic activity, limiting its use as an antibiotic to topical applications. In a wide variety of environments, the GS molecule exists as a very stable amphiphilic antiparallel β-sheet structure with a polar and a non-polar surface. Moreover, the large number of structure–activity studies of GS analogs which have been carried out indicate that this 'sidedness' structure is required for its antimicrobial action. In this review, we summarize both published and unpublished biophysical studies of the interactions of GS with lipid bilayer model and with biological membranes. In general, these studies show that GS partitions strongly into liquid–crystalline lipid bilayers in both model and biological membranes, and seems to be located primarily in the glycerol backbone region below the polar headgroups and above the hydrocarbon chains. The presence of GS appears to perturb lipid packing in liquid–crystalline bilayers and GS can induce the formation of inverted cubic phases at lower temperatures in lipids capable of forming such phases at higher temperature in the absence of peptide. The presence of GS at lower concentrations also increases the permeability of model and biological membranes and at higher concentrations causes membrane destabilization. There is good evidence from studies of the interaction of GS with bacterial cells that the destruction of the integrity of the lipid bilayer of the inner membrane is the primary mode of the antimicrobial action of this peptide. The considerable lipid specificity of GS for binding to and destabilization of lipid bilayer model membranes indicates that the design of GS analogs with an improved antimicrobial potency and a markedly decreased toxicity for eukaryotic cell plasma membranes should be possible. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Gramicidin S; Antimicrobial peptide; Lipid bilayer; Model membrane; Biological membrane; Membrane permeability; Membrane lysis; Differential scanning calorimetry; Nuclear magnetic resonance spectroscopy; Electron spin resonance spectroscopy; Fluorescence spectroscopy; Fourier transform infrared spectroscopy

**Contents**

## 1. Structure and conformation of GS

Gramicidin S (GS) is a cyclic decapeptide of primary structure [cyclo-(Val-Orn-Leu-D-Phe-Pro)$_2$] (see Fig. 1) first isolated from *Bacillus brevis* [1] and is one of a series of antimicrobial peptides produced by this microorganism (see [2,3]). GS generally exhibits considerable specificity for the killing of Gram-positive bacteria when assayed on solid growth media (see [2,3]), but in liquid media, this peptide shows potent antibiotic activity against a broad spectrum of both Gram-negative and Gram-positive bacteria as well as several pathogenic fungi [4,5]. Unfortunately, GS is rather non-specific in its actions and exhibits appreciable hemolytic as well as antimicrobial activity, thus restricting the use of GS as an antibiotic to topical applications [2,3]. However, recent work has shown that structural analogs of GS can be designed with markedly reduced hemolytic activity and enhanced antimicrobial activity, suggesting the possibility that appropriate GS derivatives may be used as potent oral or injectable broad-spectrum antibiotics [4,5].

GS has been extensively studied by a wide range of physical techniques (see [2,3]) and its three-dimensional structure is shown in Fig. 1. In this minimum energy conformation, the two tripeptide sequences Val-Orn-Leu form an antiparallel β-sheet terminated on each side by a Type II′ β-turn formed by the two D-Phe-Pro sequences. Four intramolecular hydrogen

bonds, involving the amide protons and carbonyl groups of the two Leu and two Val residues, stabilize this rather rigid structure. Note that the GS molecule is amphiphilic, with the two somewhat polar and positively charged Orn side-chains and the two D-Phe rings projecting from one side of this molecule, and the four hydrophobic Leu and Val side-chains projecting from the other side. A number of studies have shown that this 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 often alter protein conformation [2,3]. This conformational invariance of GS can be a major advantage in certain biophysical studies, as illustrated later in this review.

Considerable evidence exists that the principal target of GS is the lipid bilayer of bacterial or erythrocyte membranes (see [2,3]). In this review, we summarize the biophysical studies of the interaction of GS with lipid bilayer model and with bacterial inner membranes, with the goal of obtaining insight into the mechanism of action of this well-studied and potentially therapeutically useful antimicrobial peptide.

## 2. Structure–activity relationships for GS

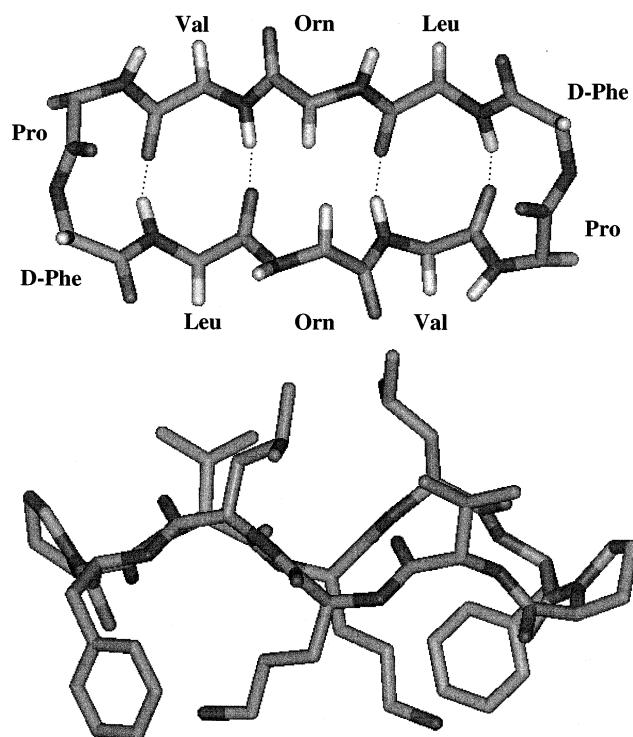There have been a large number of studies of the

Fig. 1. The structure and conformation of GS. The upper 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 β-sheet region. The lower 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.

relationship between the chemical structure and conformation of GS analogs and their antimicrobial activity, and in some cases also of the interactions of these analogs with lipid bilayer model and with biological membranes. In fact, GS is by far the best studied antimicrobial peptide in this regard. A brief summary of the major results of these extensive studies is provided below. For a detailed account of this work, the reader is referred to the excellent reviews by Izumiya et al. and Waki and Izumiya [2,3].

The basic character of the two Orn residues seems to be essential for the antimicrobial activity of GS. Thus the replacement of both the Orn residues with various neutral or acidic amino acids results in a complete loss of activity. Moreover, even the replacement of a single Orn residue by a neutral amino acid markedly reduces activity. However, the replacement of both Orn residues by the strongly basic Arg or Lys residues does not reduce antimicrobial effectiveness, whereas replacement of the two Orn residues by the weakly basic His residues results in a major reduction in activity, as does acylation of the δ-amino groups or Orn or Lys. In addition, the presence of two basic amino acid residues on the same face of the peptide molecule is required for maximal antimicrobial potency.

The hydrophobic character of the two Leu and two Val residues which make up the other face of the GS molecule are also important for antimicrobial activity. Thus the simultaneous replacement of these four amino acid residues by hydrophilic amino acids or by aliphatic amino acids with smaller side chains results in complete loss of antimicrobial activity. The replacement of one Leu and one Val residue by aromatic Phe residues reduces activity slightly, but replacement of all four of these hydrophobic residues by four Phe residues results in a major loss of activity. Similarly, the replacement of one Leu and one Val, or of both Leu or both Val residues, by two Gly residues produces a substantial decrease in activity, as does replacement on the two Leu residues by Ala or Val or by aromatic Phe residues. However, the replacement of one or both Leu residues by methyl Leu results in retention of full antimicrobial activity.

The D-Phe residues play a specific role in the maintenance of the β-sheet structure of GS by participating in the two type II′-β-turns at the D-Phe-Pro sequences. As might be expected, the replacement of the two D-Phe residues by L-Phe or other L-amino acid residues destabilizes the β-sheet conformation and leads to a complete loss of antimicrobial activity. However, the D-Phe residues can be replaced by other aromatic or bulky aliphatic D-amino acids without loss of activity, but replacement by less hydrophobic or by hydrophilic D-amino acid residues leads to a progressive loss of antimicrobial potency. Similarly, the Pro residues can be replaced by a variety of amino acids with full retention of activity, provided that such replacements do not alter the native conformation greatly.

Virtually all of the results of the structure–activity studies reported to date can be explained by the 'sidedness' hypothesis originally proposed by Schwyzer [6] and by Kato and Izumiya [7]. This hypothesis states that the amphiphilic nature of the GS molecule, resulting from the presence of two fairly

hydrophilic, positively charged amino acid residues on one side of this discoid-like molecule, and four hydrophobic residues on the other side, as shown in Fig. 1, is crucial for the manifestation of anti-microbial activity. Thus, any amino acid substitution which reduces the 'sidedness' of GS, either directly or indirectly by a loss of the stable β-sheet structure, will reduce the antimicrobial potency of this peptide.

## 3. Interactions of GS with lipid bilayer model membranes

### 3.1. Effects of GS on lipid thermotropic phase behavior

The effect of membrane-associated peptides on lipid thermotropic phase behavior can provide useful information on the nature of the interactions between these two important classes of biological membrane constituents and on the general topology of the peptide relative to the lipid bilayer [8,9]. The most powerful technique for such studies is probably differential scanning calorimetry (DSC), a convenient and sensitive non-perturbing thermal method which provides reliable thermodynamic information on the effects of peptides on the thermotropic phase behavior of the host lipid bilayer [10]. DSC has been applied to study the interactions of GS with several phospholipid systems, especially with zwitterionic phosphatidylcholine (PC) systems. The results of these studies, which are not always consistent, are summarized below.

The first calorimetric study of the effect of GS on phospholipid thermotropic phase behavior was the LS-DSC study of Pache et al. [11], who studied the effect of GS on the gel to liquid–crystalline phase transition of zwitterionic dipalmitoylphosphatidylcholine (DPPC) large multilamellar vesicles (MLVs). These workers reported that at a DPPC/GS molar ratio of 10:1, the phase transition temperature ($T_m$) of the DPPC bilayer was shifted slightly downward and the DSC endotherm was broadened slightly, while at a molar ratio of 1:1 the $T_m$ was reduced about 5°C and the cooperativity of the phase transition was reduced modestly. In both cases the addition of GS was reported to *not affect* the phase

transition enthalpy ($\Delta H$) nor to induce multicomponent endotherms. In a later LS-DSC study, Wu et al. [12] also reported a downward shift in the $T_m$ of DPPC MLVs by 2–3°C at DPPC/GS concentrations of 10:1 to 5:1, but found that the cooperativity of the chain-melting phase transition was not affected. Moreover, again only single-component DSC endotherms were observed after GS addition. However, these investigators reported that the addition of increasing quantities of GS *substantially increased* the $\Delta H$ over the range of GS concentrations studied. Nakagaki et al. [13], again utilizing LS-DSC, then reported that GS depressed the $T_m$ of DPPC MLVs by a maximum of about 5°C, but in this case the cooperativity of the gel to liquid–crystalline phase transition was reported to be considerably reduced and two-component endotherms were observed at intermediate GS concentrations (from DPPC/GS molar ratios of about 15:1 to 5:1). These workers also reported that the total $\Delta H$ (areas under both peaks, when present) was *essentially constant* with GS concentration. Finally, using HS-DSC, Datema et al. [14] reported that at a DPPC/GS ratio of 6:1, the $T_m$ of the DPPC MLVs was reduced by about 1.5°C, the cooperativity of the main phase transition was reduced only slightly, and only a single, symmetric DSC endotherm was present. However, at a *lower* GS concentration (DPPC/GS molar ratio of 13:1), the phase transition was reported to be *increased* in temperature, was *broadened considerably* and appeared to have a *lower temperature shoulder*. Unfortunately, $\Delta H$ values were not reported in this study. Thus, although there seems to be a general consensus in these four studies that GS generally decreases the $T_m$ of DPPC bilayers, the reported effects of GS addition on transition cooperativity and enthalpy vary considerably, and both single- and multicomponent DSC endotherms have been reported at comparable GS concentrations. Possible reasons for these discrepancies in results are discussed below. One should note, however, that both diphenylhexatriene (DPH) fluorescence polarization [15] and deuterium nuclear magnetic resonance ($^2$H-NMR) [14,16] spectroscopic studies confirm that, at least at fairly high concentrations (DPPC/GS molar ratios ranging from 2.5:1 to 6:1, respectively), GS broadens and lowers the $T_m$ of the gel to liquid–crystalline phase transition in DPPC

MLVs. Interestingly, Nakagaki and coworkers [13] also reported that GS generally affected the thermotropic phase behavior of MLVs of the zwitterionic phospholipid dipalmitoylphosphatidylethanolamine (DPPE) in a similar manner to zwitterionic DPPC MLVs, by lowering the $T_m$ and reducing the cooperativity of the chain-melting phase transition, and by inducing multicomponent endotherms. However, in contrast to the lack of a clear effect of GS on the $\Delta H$ of the DPPC phase transition reported by these workers, a substantial *increase* in the $\Delta H$ of the DPPE phase transition was noted as GS concentrations increased. Moreover, at comparable concentrations, GS lowered the $T_m$ of DPPE bilayers to a greater extent than for DPPC bilayers.

Several LS-DSC studies of the thermotropic phase behavior of GS-containing dimyristoylphosphatidylcholine (DMPC) MLVs have also been published. Susi et al. [17], investigating a 25% w/w peptide–lipid mixture (corresponding to a DMPC/GS molar ratio of about 6.5:1), reported that the sharp endotherm of DMPC alone centered at 23°C was replaced by two broader peaks centered at 12.0 and 16.2°C upon GS addition. Moreover, the total $\Delta H$ was *reduced markedly* (about 80%) by the presence of this comparatively high concentration of peptide. Moreover, the DSC endotherms coincided well with the DMPC hydrocarbon chain-melting phase transition monitored in this mixture by Raman spectroscopy, which occurred over the temperature range 8–20°C. Although Wu et al. [12] also reported the induction of multi-component DSC endotherms at DMPC/GS molar ratios of 25:1 and 5:1, the decrease in the $T_m$ of these components was reported to be only 4–6°C, even at the highest peptide concentration tested. Moreover, the total $\Delta H$ was reported to *increase substantially* as GS concentration increased. Therefore, although these two studies agree that GS decreases the $T_m$ and cooperativity of the gel to liquid–crystalline phase transition of DMPC MLVs and induces the multicomponent DSC endotherms, the magnitude of the $T_m$ decreases found differs significantly in these studies, and the reported $\Delta H$ trends are in opposite directions!

We [18] have recently investigated the effects of GS on the thermotropic phase behavior of large MLVs of the zwitterionic phospholipids DMPC and DMPE and of the anionic lipid dimyristoylphosphatidylgly-
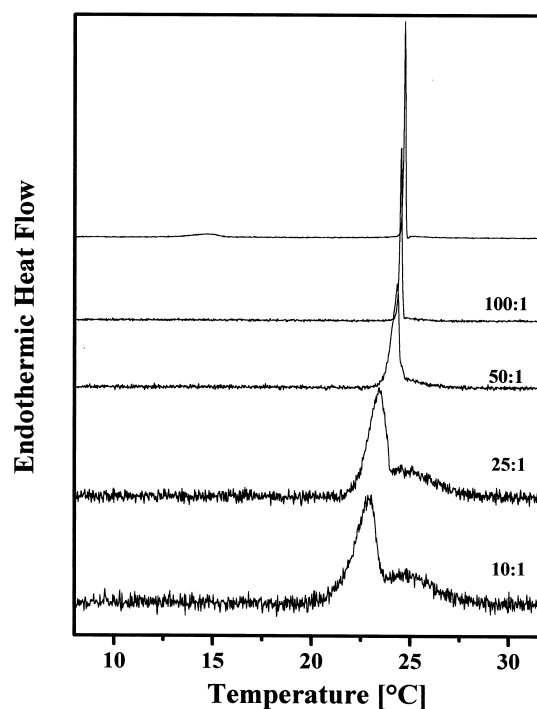


Fig. 2. High-sensitivity DSC heating scans illustrating the effect of the presence of increasing quantities of GS on the thermotropic phase behavior of DMPC MLVs. The top scan is of DMPC alone and the DMPC/GS molar ratios of the lower scans are indicated on the figure itself.

cerol (DMPG) by HS-DSC, utilizing a wide range of GS concentrations (phospholipid/GS molar ratios of 1000:1 to 10:1). We found that small amounts of GS decreased the temperature and enthalpy of the pretransition and that the pretransition was abolished entirely in DMPC vesicles having lipid/peptide ratios of 100:1 or less. Moreover, the presence of increasing quantities of GS also resulted in the induction of a two-component main phase transition, with a more cooperative, lower temperature endotherm superimposed over a less cooperative, higher temperature endotherm (Fig. 2). The $T_m$ and relative $\Delta H$ of the sharper component decreased with increasing GS concentration while those of the broader component increased and the cooperativity of both components of the chain-melting phase transition also decrease with increases in peptide concentration relative to DMPC alone. However, the GS-induced decreases in the phase transition temperature of the sharp component, and increase in the temperature of the broad

component, were slight (2–3°C and about 1°C, respectively) and the total $\Delta H$ of chain melting was *reduced* by only 10–15%, even at a phospholipid/peptide ratio of 10:1. These results indicate that the presence of GS produces only a weak overall destabilization of the gel state of DMPC bilayers, even after maximizing GS-DMPC interactions by multiple cycling through the gel to liquid–crystalline phase transition.

In contrast, if GS was incorporated into dimyristoylphosphatidylethanolamine (DMPE) MLVs at temperatures above, but near to, the gel to liquid–crystalline phase transition temperature, we found essentially no effect on the thermotropic phase behavior upon heating, even at high GS concentrations. However, if GS-containing DMPE vesicles were exposed to temperatures well above the gel to liquid–crystalline phase transition temperature, increasing quantities of GS lowered the temperature, enthalpy and cooperativity of the chain-melting phase transition of DMPE slightly when DSC cooling curves were run. Moreover, upon subsequent reheating, the characteristic effects of the presence of the peptide on the phase behavior of DMPE vesicles were retained. However, the effects of GS addition on the temperature, enthalpy and cooperativity of the main phase transition observed upon cooling and subsequent reheating were small, although the shift in the $T_m$ of the broad component of the DSC endotherm to lower temperatures was more pronounced than in DMPC bilayers at comparable GS concentrations, even at the highest peptide phospholipid ratio tested, in agreement with the results of Nakagaki and coworkers for DPPE bilayers [13]. However, in contrast to their results with DPPE MLVs, we observed a slight decrease in the enthalpy of the chain-melting phase transition in DMPE bilayers. Our findings indicate that the presence of GS produces only a modest destabilization of the gel state of DMPE bilayers and then only if the GS containing vesicles are first exposed to high temperatures. However, the interaction of GS with DMPE bilayers at high temperatures significantly reduced the temperature of the lamellar liquid–crystalline/reversed hexagonal ($L_\alpha/H_{II}$) phase transition, indicating that structurally significant interactions between GS and a sufficiently fluid phase of DMPE can occur, and that these interactions do persist after cool-
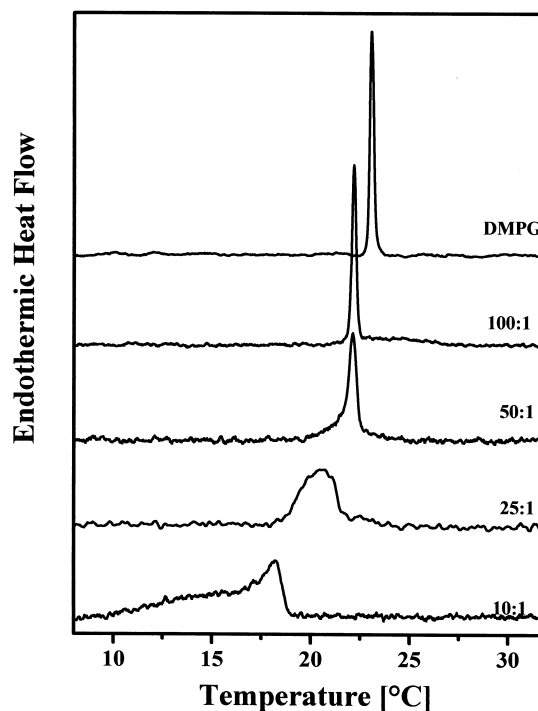


Fig. 3. High-sensitivity DSC heating scans illustrating the effect of the presence of increasing quantities of GS on the thermotropic phase behavior of DMPG MLVs. The top scan is of DMPG alone and the DMPG/GS molar ratios of the lower scans are indicated on the figure itself.

ing to temperatures below the main phase transition temperature.

We [18] found that the interaction of GS with DMPG MLVs had a major effect on the thermotropic phase behavior of this anionic phospholipid. Specifically, the presence of increasing quantities of GS reduced the temperature and enthalpy of the pretransition, which appeared to be abolished entirely at lipid/peptide ratios of 500:1 or less. Moreover, at low concentrations of peptide, two endotherms were clearly present in the DSC healing thermograms, a more cooperative, relatively energetic endotherm centered at 22°C and a less cooperative, less energetic endotherm centered near 27°C (Fig. 3). As the GS concentration increased, the initially higher temperature endotherm decreased in temperature and cooperativity, but became relatively more energetic as compared to the lower temperature, more cooperative endotherm. However, at higher peptide concentrations, both endotherms decreased in temperature,
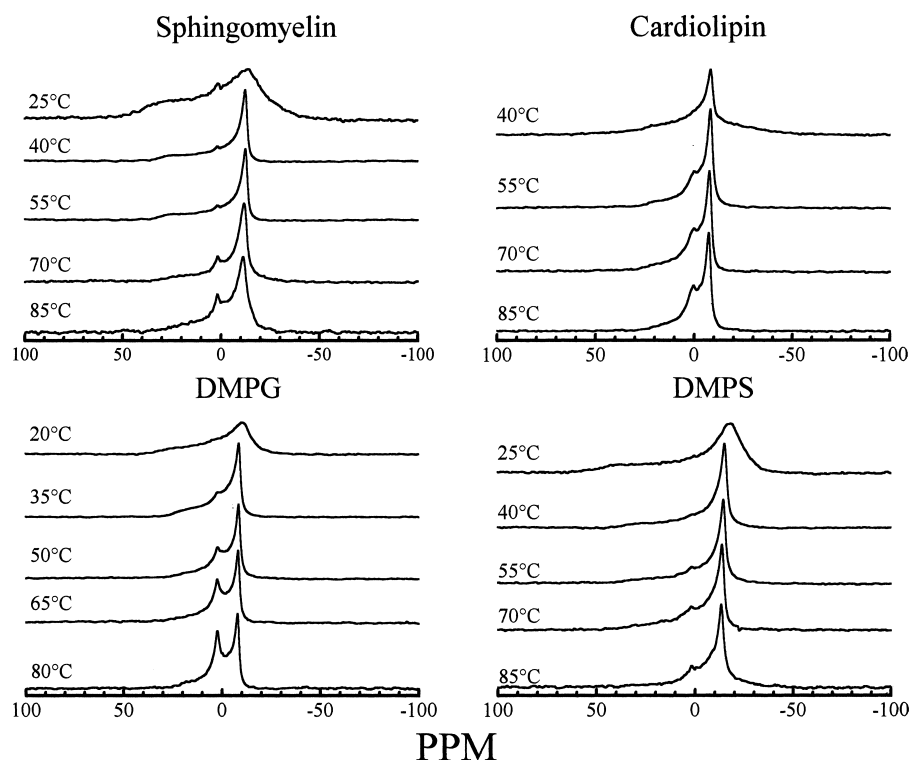
Fig. 4. Proton-decoupled $^{31}$P-NMR spectra of binary mixtures of GS with various phospholipids. The spectra were acquired at the temperatures indicated at a phospholipid/GS molar ratio of 25:1.

but the less cooperative transition, which became increasingly more prominent, was now centered at a lower temperature than the more cooperative transition. Moreover, the total enthalpy associated with both transitions decreased substantially with increasing peptide concentration, particularly at higher peptide concentrations. If one assumes that the sharp endotherm is due to the chain-melting of DMPG domains relatively poor in peptide and the broad endotherm is due to the presence of domains of DMPG enriched in peptide, then it appears that at lower concentrations GS stabilizes the gel state of DMPG bilayers, as might be expected for a positively charged peptide. However, at higher GS concentrations, the presence of GS appears to destabilize both the relatively peptide-rich and peptide-poor domains of DMPG vesicles. The effects of GS on the temperature, enthalpy and cooperativity of the gel to liquid–crystalline phase transition of DMPG vesicles were much larger than those observed in DMPE or DMPC vesicles at comparable peptide concentrations. We have also found that the presence of GS protects DMPG vesicles from the chemical hydroly-

sis induced by their repeated exposure to high temperatures.

In summary, our calorimetric results indicate that GS interacts more strongly with anionic than with zwitterionic phospholipid bilayers, probably because of the more favorable net attractive electrostatic interactions between the positively charged peptide and the negatively charged polar headgroup in such systems. Moreover, at comparable reduced temperatures, GS appears to interact more strongly with zwitterionic DMPC than with zwitterionic DMPE bilayers, probably because of the more fluid character of the former system. The reduced interactions of GS with DMPC bilayers containing cholesterol recently reported by us supports this suggestion [19]. In addition, the general effects of GS on the thermotropic phase behavior of zwitterionic and anionic phospholipids suggest that it is located at the polar/apolar interface of liquid–crystalline bilayers, where it interacts primarily with the polar headgroup and glycerol-backbone regions of the phospholipid molecules and only secondarily with the lipid hydrocarbon chains. Finally, the considerable lipid specificity
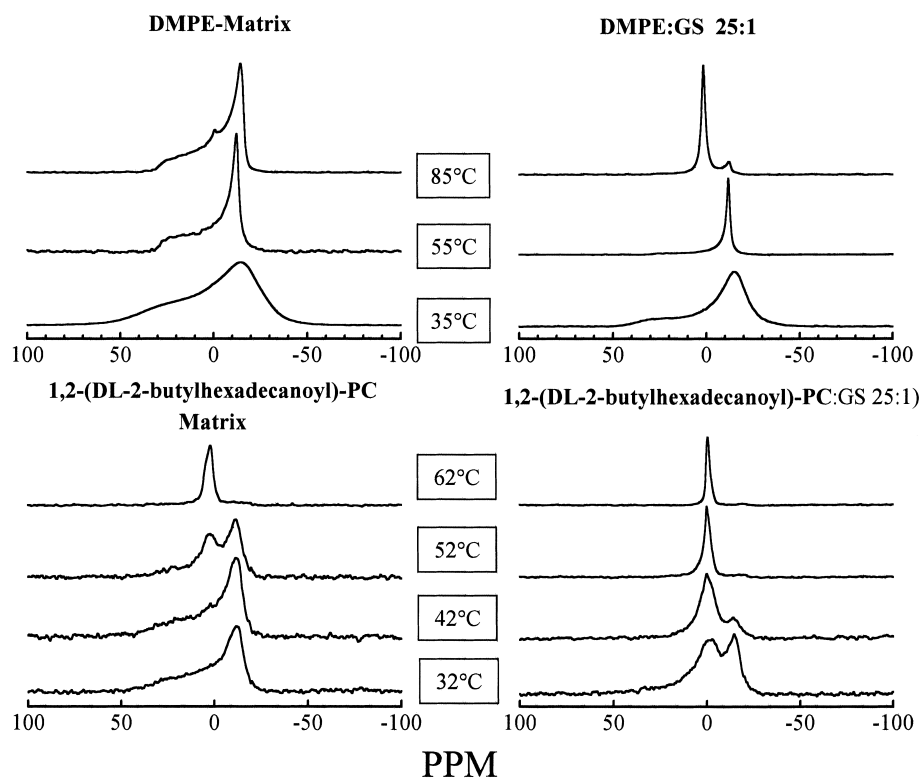
Fig. 5. Proton-decoupled ^{31}P-NMR spectra of DMPE and 1,2-(DL-2-butylhexadecanoyl)-PC MLVs obtained in the absence or presence (phospholipid/GS molar ratio 25:1) of GS at the temperatures indicated on the figure itself. The latter phospholipid is a non-lamellar-phase preferring phosphatidylcholine (see [41]).

of GS interactions with phospholipid bilayers may prove useful in the design of peptide analogs with stronger interactions with microbial as opposed to eukaryotic membrane lipids.

Our recent HS-DSC studies of the effect of GS on the thermotropic phase behavior of DMPC, DMPE and DMPG MLVs have revealed that their calorimetric behavior depends on both the method of preparation and thermal history of the sample [18]. For example, equilibration of the samples at low temperatures before initiating a DSC heating scan, and several cycles through the phase transition region, are generally required to produce consistent and reproducible behavior. As well, as mentioned above, it was necessary to heat DMPE vesicles to a temperature well above their phase transition temperature to see an effect of GS on the main phase transition of this phospholipid. These factors, plus the use of LS-DSC and high scan rates in the earlier studies, may account for some of the differences in the results reported in the studies summarized above.

However, some of the very different findings reported in previous studies suggest other problems yet to be resolved.

In addition to affecting the thermodynamic parameters of the gel to liquid–crystalline phase transition of phospholipid bilayers, we have recently shown the GS can also alter the lamellar/non-lamellar phase preferences of some phospholipid and glycolipid molecular species [20]. Specifically, we examined the interactions of GS with a variety of single-component lipid bilayers, and with membrane polar lipid extracts of *Acholeplasma laidlawii* B and *Escherichia coli*, by ^{31}P-nuclear magnetic resonance (^{31}P-NMR) spectroscopy and X-ray diffraction. For mixtures of GS with lipids, such as phosphatidylcholine, phosphatidylserine, cardiolipin, and sphingomyelin, axially symmetric ^{31}P-NMR powder patterns were observed throughout the entire temperature range examined (0–90°C) and there was little evidence for significant destabilization of the lipid bilayer with respect to non-lamellar phases (see Fig. 4). With mix-

tures of GS with either phosphatidylethanolamine, phosphatidylglycerol, or a non-lamellar phase-forming phosphatidylcholine, axially symmetric ^{31}P-NMR powder patterns were also observed at low temperatures. However, at high temperatures, an isotropic component was observed in their ^{31}P-NMR spectra, and the relative intensity of this component increased significantly with temperature and with GS concentration(see Fig. 5). Once formed at high temperatures, this isotropic component exhibited a marked cooling hysteresis and in most cases disappeared only when the sample was recooled to temperatures well below the lipid hydrocarbon chain-melting phase transition temperature. We also showed that GS induces the formation of isotropic components in the ^{31}P-NMR spectra of heterogeneous lipid mixtures such as occur in *A. laidlawii* B and *E. coli* membranes. These observations suggest that GS induces the formation of cubic or other three dimensionally ordered inverted non-lamellar phases when it interacts with some types of lipid bilayers, a suggestion strongly supported by our X-ray diffraction studies. Moreover, we found that the capacity of GS to induce the formation of such phases increases with the intrinsic non-lamellar phase-preferring tendencies of the lipids with which it interacts, probably by producing localized increases in membrane monolayer curvature stress. The latter effect could be part of the mechanism through which this peptide exhibits its antimicrobial and hemolytic activities.

We have also recently demonstrated that the presence of cholesterol can modulate the tendency of GS to induce the reversed cubic phase in phospholipid bilayers capable of forming such phases at high temperatures (unpublished results). For example, dipalmitoylelaidoyl-phosphatidylethanolamine (DPEPE) in the absence of peptide exhibits a lamellar gel to liquid–crystalline phase transition at 20.7°C and a lamellar liquid–crystalline to reversed hexagonal phase transition at 92.5°C. Thus this phospholipid exists exclusively in the lamellar liquid–crystalline phase over the temperature range from 25 to 70°C, as shown by the characteristic bilayer ^{31}P-NMR spectra presented in Fig. 5. The addition of GS (DPEPE/GS molar ratio 25:1) resulted in the induction of a major isotropic peak near +2 ppm in the ^{31}P-NMR spectra at 55°C, due to the formation of a reversed cubic phase, and at 70°C this cubic phase
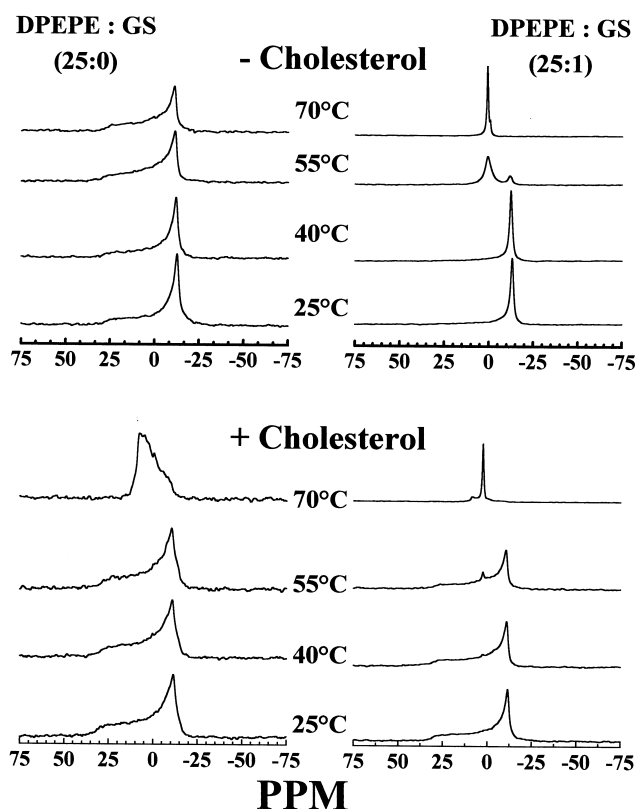


Fig. 6. Proton-decoupled ^{31}P-NMR spectra of DPEPE, with or without 25 mol% cholesterol, in the presence and absence of GS (DPEPE/GS molar ratio 25:1), acquired at the temperatures indicated on the figure itself.

completely replaced the bilayer phase. However, with DPEPE bilayers containing 25 mol% cholesterol, the addition of the same quantity of GS produced a largely bilayer ^{31}P-NMR spectra at 55°C, and some bilayer phase contribution also persisted at 70°C. Thus, the presence of cholesterol reduced the tendency of GS to induce the inverted cubic phase in DPEPE bilayers at temperatures where only the lamellar liquid–crystalline phase would normally exist in the absence of peptide. This bilayer-stabilizing effect must be due to an indirect action of cholesterol in inhibiting the association with and/or penetration of GS into phospholipid bilayers, since the addition of cholesterol itself *destabilized* the lamellar phase in pure DPEPE bilayers, as does GS itself (Fig. 6). Indeed, Fourier transform infrared (FTIR) spectroscopic evidence for this indirect effect of cholesterol on GS interactions with phospholipid bilayers will be presented below.

## 3.2. Effects of GS on the organization and dynamics of lipid bilayer membranes

One of the earliest biophysical studies of the effects of GS addition on the physical properties of phospholipid vesicles was that of Finer et al. [21], who utilized ^{1}H-NMR and electron spin resonance (ESR) spectroscopy to investigate the effect of high concentrations of GS on the mobility of small unilamellar vesicles (SUVs) and MLVs of egg PC in the liquid–crystalline state. These workers reported that GS does not alter the line broadening or signal area of the NMR spectra of sonicated vesicles, even at an egg PC/GS molar ratio of 1.3:1. However, the addition of this concentration of GS to large multilamellar vesicles converted the broad, featureless NMR spectra into a high resolution spectra, due at least in part to conversion of the large, slowly tumbling MLVs to small, freely tumbling GS-phospholipid particles. Moreover, it was also reported that the ESR spectra of sonicated and unsonicated egg PC vesicles was unchanged by GS addition. Thus, it would appear from this study that GS binding does not affect the motional properties of egg PC molecules to which the peptide is bound, although this early investigation would not be very sensitive to modest changes in phospholipid organization and dynamics. Similar findings were later reported by Pache et al. [11], again using ^{1}H-NMR and ESR methods, although a careful inspection of the published spectra reveals some small effects resulting from GS addition.

Zidovetski et al. [16] later utilized ^{2}H- and ^{31}P-NMR spectroscopy to investigate the effect of GS on the hydrocarbon chain orientational order profile and polar headgroup orientation of chain-perdeuterated DMPC MLVs in the gel and liquid–crystalline states. These workers reported that GS addition to gel-state DMPC MLVs produced no detectable changes in hydrocarbon chain or polar headgroup organization, suggesting the GS is excluded from DMPC gel-state bilayers, even at relatively high GS concentrations. In contrast, GS produced a concentration-dependent decrease in hydrocarbon chain orientational order in the liquid–crystalline state, particularly in the region of the glycerol backbone, and a change in the orientation of the polar headgroup relative to the bilayer normal. These results indicate

that above the phase transition temperature of various GS/DMPC mixtures, this peptide interacts with both the polar headgroup and the hydrocarbon chains of the DMPC molecules. These workers thus suggested an interfacial location of GS in which the positively charged ornithine residues interact electrostatically with the negatively charged phosphates and the non-polar amino acids residues of the peptide penetrate into the hydrocarbon core of the DMPC bilayer, disordering the hydrocarbon chains. Although the motional rates of the DMPC hydrocarbon chains and polar headgroups were not measured in this study, it would be unusual if these fairly pronounced changes in the orientational order of these two regions of the phospholipid bilayer were not also reflected in altered phospholipid dynamics.

There have been three other studies of the effect of GS on the organization of the hydrocarbon core of phospholipid bilayers. Susi et al. [17], utilizing Raman spectroscopy, reported that GS (molar ratio 6.5:1) disordered the hydrocarbon chains of DMPC below the broadened and reduced gel to liquid–crystalline phase transition but had no effect at temperatures above the phase transition. In contrast, Katsu et al. [15], using DPH fluorescence polarization spectroscopy, found that GS (molar ratio 2.5:1) has no effect on hydrocarbon chain organization in DPPC bilayers either below or above the phase transition region. Finally, Yagi et al. [22], also using DPH fluorescence polarization spectroscopy, reported that GS at a phospholipid/peptide molar ratio of 10:1 slightly disordered DPPC SUVs in the liquid–crystalline state. Most of these results, and the ^{2}H-NMR findings of Zidovetski and coworkers [16], are clearly inconsistent with one another. In our view, the ^{2}H-NMR results are likely to be the more reliable, particularly in the liquid–crystalline state, but additional work will be required to definitively resolve these experimental discrepancies.

Mihailescu and Horváth [23] have recently studied the association of spin-labeled DMPC and dimyristoylphosphatidylserine (DMPS) molecules with GS by ESR spectroscopy in pure or mixed MLVs. These workers reported the existence of about six molecules of motionally restricted phospholipid associated with each GS molecule between 30–40°C, although this number was somewhat dependent on temperature. Moreover, the boundary lipid population of phos-

pholipid in mixed phospholipid vesicles contained a slight excess of the anionic DMPS as compared to the zwitterionic DMPC ($K$ (boundary/bulk lipid) for DMPS reported as $1.18 \pm 0.08$ as compared to 1.00 for DMPC), and the localization in the boundary lipid of spin-labeled stearic acid ($K$ of $2.08 \pm 0.10$) was even stronger, suggesting a preference of GS for binding anionic lipids. However, as phospholipids have two hydrocarbon chains per molecule where stearic acid of course has only one, whether or not a $K$ value of about 2 for the free fatty acid indicates any specificity for GS binding is unclear. Moreover, if GS binds two molecules of DMPS tightly by electrostatic interactions, but had equal binding affinities for DMPC and DMPS at the hydrophobic binding sites, a $K$ value near 2.0 would be expected. Moreover, the spin-labeled cationic anesthetic procaine also showed preferential association with this peptide ($K$ of $1.28 \pm 0.12$), indicating that factors other than charge may be important. These authors suggested that the two cationic Orn residues of the GS molecule are involved in phospholipid binding, although hydrophobic interactions must also be involved in lipid association in bilayer membranes.

We have some reservations about the conclusions of the ESR spectroscopic study of Mihailescu and Horváth, in that the motionally restricted lipids were only detected in the temperature range 30–40°C, and subtraction of ESR spectra at a temperature 5°C lower than the experimental spectra was employed to generate a difference spectra, supposedly due entirely to the motionally restricted boundary lipid component. However, our HS-DSC results indicate that at a DMPC/GS molar ratio of 10:1, the DSC peak due to the melting of GS-associated phospholipid extends from about 20 to nearly 30°C, and would presumably extend over an even greater temperature range at the lower lipid/peptide molar ratio of 7:1 usually employed by these workers. Since the proportion of GS-associated gel-like phospholipid would be expected to progressively decrease with temperature over the range 25–35°C, these authors may be detecting lipid which is motionally restricted due to its physical state rather than to its binding to GS per se. Moreover, above this temperature range, all of the lipid in GS-containing vesicles would be in the liquid–crystalline state, perhaps accounting for

the loss of the 'boundary lipid' signal. However, Beyer and Huber [24], utilizing ^{1}H-NMR spectroscopy, have reported that in GS/octaethylene glycol mono-*n*-dodecyl ether mixed micelles, about five non-ionic detergent molecules are bound in the 'first shell' surrounding each GS molecule. Moreover, based on the relative areas of the GS molecule, arranged with its ring system parallel to the bilayer plane, and the relative cross-sectional areas of phospholipid molecules, a binding stoichiometry of five or six phospholipid molecules per peptide molecule is predicted, so perhaps these ESR spectroscopic results are valid after all.

## 3.3. Partitioning and localization of GS in lipid bilayer membranes

Yagi et al. [22] utilized the negatively charged hydrophobic fluorescent probe 8-anilino-1-naphthalene sulfonic acid (ANS) to indirectly monitor GS binding to DPPC SUVs in the liquid–crystalline state. Since binding of the positively charged GS to zwitterionic DPPC bilayers should produce a positive membrane surface potential, the incremental binding of ANS to SUVs should be proportional to the amount of GS present. Indeed, the addition of GS (molar ratio of DPPC/GS 125:1) produced a rapid increase in ANS binding to the phospholipid vesicles, while addition of peptide in the absence of DPPC SUVs did not change the fluorescence intensity of the probe. However, the degree of GS binding was not quantitated in this study. These workers did report that 70% of added GS was bound to DPPC MLVs when the phospholipid/peptide ratio was 5000:1. However, these latter measurements were made at 28°C, where DPPC exists in the gel state. Moreover, since the volume of the aqueous phase in this latter experiment was not specified, a phospholipid/water partition coefficient for GS cannot be calculated. However, it seems clear that more GS must be bound to the lipid than to the water phase, even in these gel-state DPPC MLVs.

Higashijima and Miyazawa [25] utilized ^{1}H-NMR to study the interaction of GS with SUVs of egg PC or egg PC/egg PS (molar ratio 9:1) at very low molar ratios of lipid/peptide (0.25:1.00 to 1.00/1.00) at 23°C, well above the gel to liquid–crystalline phase transition temperature of these phospholipids. They
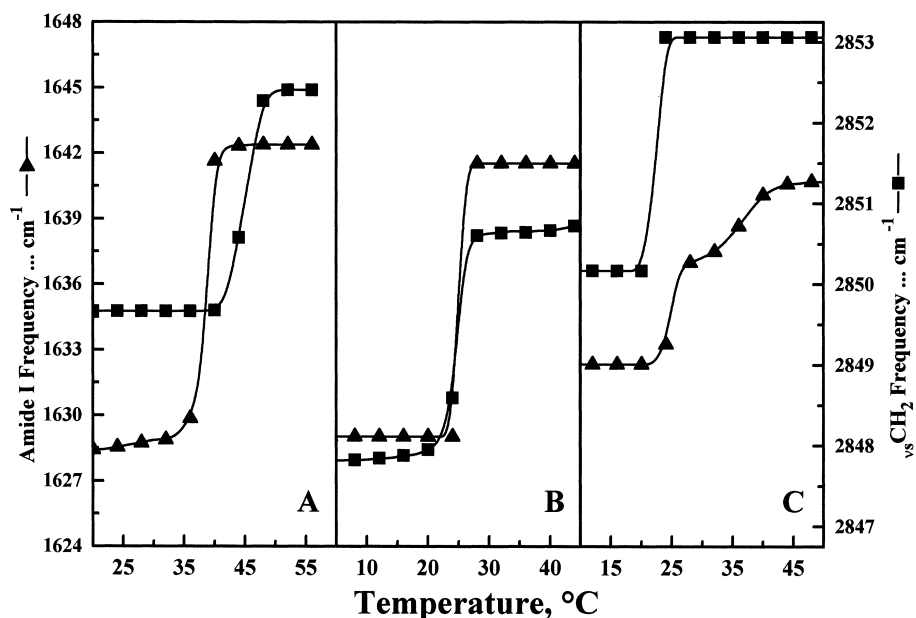
Fig. 7. Temperature-dependent changes in the peak frequencies of the phospholipid hydrocarbon chain CH$_2$ symmetric stretching (■) and peptide amide I (▲) infrared absorption bands of binary mixtures of GS with tetramyristoyldiphosphatidylglycerol (A), dilauroyl-phosphatidylethanolamine (B) and P-O-ethyl-DMPC (C) bilayers (phospholipid/GS molar ratios 25:1). These mixtures illustrate the behavior of GS in anionic, in zwitterionic or uncharged, and in cationic lipid bilayers, respectively.

report that all of the N–H proton resonances (and aromatic ring resonances) of GS were progressively broadened upon the addition of phospholipid vesicles, with the broadening of the main ring N–H protons being more pronounced than that of the side-chain NH$_3$ protons of the Orn residues. These workers presented evidence that this signal broadening was not due to an increase in solution viscosity resulting from phospholipid vesicle addition, indicating that the rate of exchange between free and vesicle-bound GS was fast on the $^1$H-NMR time scale. Interestingly, the degree of GS signal broadening at comparable lipid/peptide ratios was greater in the PC/PS than in the PC SUVs, indicating that although the presence of an anionic phospholipid is not required for GS binding, the presence of a negatively charged phospholipid does promote peptide–lipid association in the liquid–crystalline state. Again, the actual partition coefficient of GS between the phospholipid and aqueous phases was not determined.

Two groups have studied the mobility of GS in model membrane systems and have come to different conclusions about the localization of this peptide in lipid bilayers. Wu et al. [12] studied the dependence

of the lateral diffusion coefficient of a fluorescent derivative of GS (NBD-GS, or N-4-nitrobenz-2-oxa-1,3-diazole gramicidin S) on temperature in both DMPC and DPPC bilayers containing or lacking cholesterol. These workers reported that the lateral diffusion coefficient of NBD-GS was markedly reduced upon conversion of either phospholipid bilayer to the gel state, and that the addition of cholesterol induced the aggregation of GS in DPPC and particularly in DMPC bilayers. From these results and from the generally appreciable hydrophobicity of the native GS molecule, Wu and colleagues suggested that GS must penetrate at least partially into the hydrophobic core of the host lipid bilayer. In contrast, Datema et al. [14], utilizing $^2$H-NMR and partially deuterated GS, reported that the rotational diffusion of GS was insensitive to the gel to liquid–crystalline phase transition of DPPC bilayers, in contrast to the situation with transbilayer peptides [26]. This result, in conjunction with very small reduction in the temperature and enthalpy of the gel to liquid–crystalline phase transition induced by the addition of even large quantities of GS reported by these workers, led them to suggest the GS is bound only to the surface of DPPC bilayers.

We have recently utilized FTIR spectroscopy to study the interaction of GS with lipid bilayer model membranes as a function of the phase state and fluidity of a wide variety of zwitterionic, anionic, cationic and uncharged phospho- and glycolipids [27]. Since the conformation of GS does not change with temperature or with the composition of the medium in which it is dissolved, the dependence of the frequency of the amide I band arising from the structurally invariant central β-sheet region of the peptide on the polarity of its environment can be used to monitor GS interaction with, and location in, the host lipid bilayer. As shown in Fig. 7, when the frequency of the amide I band of GS associated with a phospholipid bilayer was monitored as a function of temperature, a shift upward in frequency was always observed more or less coincident with the gel to liquid–crystalline phase transition of the host lipid bilayer. However, GS began to penetrate into gel-state bilayers of anionic phospholipids at temperatures below the chain-melting phase transition, while in cationic lipid bilayers peptide penetration did not occur until temperatures above the phase transition temperature were reached. In the highly ordered gel-state bilayers characteristic of the zwitterionic phospholipid DMPE and of uncharged glycolipids, such as monoglucosyl diacylglycerol (MGDG) and diglucosyl diacylglycerol (DGDG), the frequency of the single amide I band of GS was centered near 1630 cm$^{-1}$ (Fig. 8), just as when dissolved in water, suggesting that GS was excluded from the lipid bilayer or at least bound only to the lipid polar headgroups near the bilayer surface. However, in the relatively
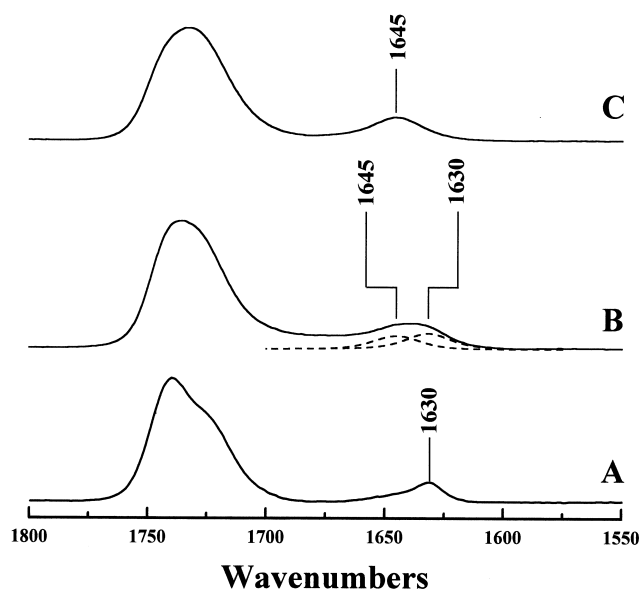


Fig. 8. FTIR spectroscopic absorbance spectra illustrating the phospholipid C=O stretching (1700–1750 cm$^{-1}$ region) and peptide amide I (1610–1660 cm$^{-1}$ region) absorption bands of gel (A and B) and liquid–crystalline (C) phospholipid bilayers. Spectrum A is of a DMPE/GS binary mixture and spectra B and C of a DMPC/GS mixture, both at a phospholipid/peptide molar ratio of 25:1.

disordered gel-state bilayers formed by the zwitterionic phospholipid DMPC and the anionic phospholipid DMPG, two peptide amide peaks were observed near 1630 and 1645 cm$^{-1}$, indicating that in this case some GS had penetrated into the less polar environment of the lipid bilayer (Fig. 8). In the liquid–crystalline state, only a single amide I band was observed, whose frequency varied from about 1640 to 1648 cm$^{-1}$ (Fig. 8 and Table 1). The absence

Table 1
Amide I band maxima of gramicidin S in lipid bilayers[a,b]

| Host lipid | Surface charge | Band maxima (cm$^{-1}$) gel phase | Band maxima (cm$^{-1}$) |
|---|---|---|---|
| DMPC | +/− | 1638[c] | 1645 |
| DMPC+cholesterol | +/− | 1628 | 1642 |
| DMPE | +/− | 1629 | 1639 |
| DMPG | − | 1636[c] | 1648 |
| P-O-ethyl DMPC | + | 1632[c] | 1641 |
| DE-MGDG | non-ionic | 1630 | 1640 |
| DE-DGDG | non-ionic | 1630 | 1641 |
| *A. laidlawii* B membrane lipids | − | 1636[c] | 1643 |

[a]Lamellar gel phase: measurements made some 10°C below the $T_m$ of the host lipid.
[b]Lamellar liquid–crystalline phase: measurements made 5°C above the $T_m$ of the host lipid.
[c]Summation of bands centered near 1630 cm$^{-1}$ and 1641–1645 cm$^{-1}$.

of an amide I band near 1630 cm$^{-1}$ indicates that essentially all of the peptide had penetrated into these fluid lipid bilayers, while the appreciable variation of the frequency of this band suggests that their depth of penetration varied somewhat. In general, the highest frequencies were observed with relatively fluid anionic phospholipids such as DMPG, intermediate frequencies with relatively fluid zwitterionic phospholipids such as DMPC, and the lowest frequencies with relatively less fluid zwitterionic phospholipids such as DMPE, by cationic phospholipids such as phosphoethyl dimyristoylphosphatidylcholine (P-*O*-E-DMPC), and by the neutral glycolipids MGDG and DGDG. Note also that the presence of 25 mol% cholesterol appeared to reduce the interaction of GS with liquid-crystalline DMPC bilayers. Thus, in general, GS associated more strongly with, and penetrated more deeply into, more disordered lipid bilayers with a negative surface charge. In the biologically relevant liquid-crystalline state, the effective polarity of the environment of GS was intermediate between that of water and a liquid hydrocarbon, corresponding roughly to the polarity experienced by the peptide when dissolved in methanol or ethanol. This finding suggests that in fluid bilayers, GS is located primarily at the interface between the lipid polar headgroups and the hydrocarbon chains near the glycerol backbone region of the lipid molecule, as suggested by the DSC [18] and $^2$H-NMR [14,16] studies reviewed earlier and the fluorescence spectroscopic studies summarized below. Our FTIR spectroscopic results also demonstrated that the phospholipid/water partition coefficient of GS is at least 1000/1 in liquid–crystalline phospholipid bilayers.

We have also investigated the interaction of GS with different phospholipid bilayers by fluorescence spectroscopy, using an analog of GS in which one of the leucine residues has been replaced by a tryptophan residue (unpublished results). This analog, termed GS10, retains appreciable antimicrobial activity. The sensitivity of tryptophan fluorescence to the environment of this amino acid residue is well established [28] and has been widely used in studies of peptide–lipid bilayer membrane interactions [29]. Specifically, the quantum yield of tryptophan fluorescence is known to increase and the wavelength of the fluorescence emission maximum to decrease from
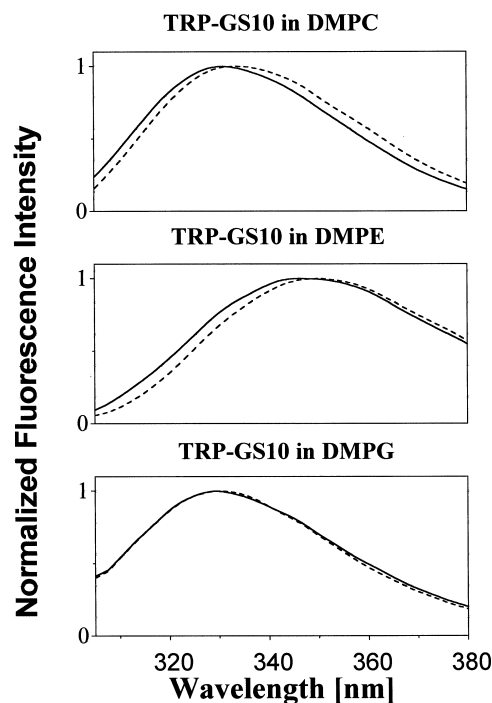


Fig. 9. Fluorescence emission spectra of tryptophan-containing GS incorporated into DMPC (top), DMPE (middle) and DMPG (bottom) MLVs of phospholipid/peptide molar ratio 25:1. Spectra recorded 10°C below the $T_m$ of the phospholipid/GS mixture (gel phase) are shown as dashed lines and spectra recorded 10°C above the $T_m$ (liquid–crystalline phase) are shown as solid lines.

roughly 360 to 310 nm in going from a polar to a non-polar environment [30]. The fluorescence emission maximum of GS10 dissolved in aqueous buffer was found to be about 350 nm and was independent of peptide concentration. As well, the fluorescence emission intensity increased linearly with peptide concentration. These results indicate that the tryptophan residues of GS10 are exposed to the water molecules when dissolved in aqueous buffers and that the peptide does not undergo changes in aggregation state under these conditions.

The interaction of GS10 with a variety of phospholipid bilayers in both the gel and liquid–crystalline states was studied and some representative results are presented in Fig. 9. In the case of the zwitterionic phospholipids DMPC and DMPE, the fluorescence emission maximum was shifted downward by 4–5 nm in going from the gel to the liquid–crystalline state, indicating a deeper penetra-

tion into a more hydrophobic environment in the more fluid bilayers. These results agree at least qualitatively with our previous FTIR spectroscopic study, which indicated that GS penetrates more extensively and/or more deeply into the less ordered DMPC as compared to the more ordered DMPE bilayers, both below and above the gel to liquid–crystalline phase transition temperature [27]. However, the emission maximum of GS10 in anionic DMPG bilayers was almost independent of phospholipid phase state and was lower than that of the same peptide in DMPC and especially in DMPE bilayers. The former result is at variance with our FTIR spectroscopic findings, where a *larger* shift in the GS amide I frequency at the chain-melting phase transition was observed with the DMPG as opposed to the DMPC or DMPE bilayer systems. However, both techniques agree that these peptides reside in a less polar environment in liquid–crystalline DMPG than in DMPC and particularly DMPE bilayers, again indicating that GS association with and/or penetration into phospholipid bilayers is facilitated by the presence of a negative surface charge and orientational disorder. Moreover, the generally 'intermediate' fluorescence emission maximum of GS10 observed in the liquid–crystalline state (about 330–345 nm) is generally compatible with a polar/non-polar interfacial location in these lipid bilayers. However, the fluorescence spectroscopic results suggest a greater range of environmental polarities for the peptide, and a generally less polar environment, than do the FTIR results. However, this could be due in part to the location of the tryptophan residue in GS10 on the hydrophobic face of the GS molecule, since in an interfacial position with the peptide ring parallel to the plane of the bilayer, this amino acid residue would likely project toward the center of the bilayer and penetrate partially into the hydrocarbon chain region. The FTIR study of GS itself, on the other hand, measures the average polarity of the environment of the peptide bonds in the central ring portion of GS molecule. Thus, this technique would be expected to sense an environment of higher polarity, as these peptide bonds would reside closer to the bilayer surface.

Semenov et al. [31] studied monolayer films of GS alone and the interaction of GS with monolayers of the zwitterionic phospholipids egg PC and DPPC

and the anionic phospholipid dipalmitoylphosphatidic acid (DPPA). These workers found that GS introduced into the aqueous subphase rapidly and quantitatively formed a monolayer film at the air/water interface. Upon lateral compression of this film, a plateau region was reported in the force/area curve. However, as the formation and persistence of this plateau region was strongly dependent on the rate of compression of the film, the significance of this finding is difficult to evaluate. At lower surface pressures, the area per molecule of GS was estimated to be 210 $\text{Å}^2$, compatible with the peptide molecule arranged at the air/water interface with the ring system parallel to the monolayer plane with the polar Orn residues projecting downward into the aqueous phase and the hydrophobic Val and Leu residues projecting upward into the air. At higher surface pressures, the area per molecule appeared to be reduced to about 145 Å, which the authors ascribed to a tilting of the ring of the GS molecule. However, this apparent decrease in molecular area in the region of the monolayer film collapse pressure could be due to a loss of peptide to the subphase or to the formation of GS multilayers. These workers also reported that GS introduced into the aqueous subphase showed strong and equal penetration into previously spread PC and PA films. The finding of an equally strong penetration of GS into zwitterionic and anionic lipids monolayers is not compatible with our previous FTIR [27] or fluorescence (unpublished) spectroscopic studies of the interactions of GS with phospholipid bilayers, nor with our unpublished studies of GS-containing DMPC and DMPG monolayers, which clearly show a preferential interaction of GS with the anionic phospholipid DMPG.

## 3.4. Effect of GS on the permeability and structural integrity of lipid bilayer membranes

There have been relatively few studies done on the effect of GS on the permeability and structural integrity of model lipid bilayer membranes, considering that the primary mode of action of this antimicrobial peptide is generally considered to be a permeabilization or disruption of the lipid phase of microbial membranes.

Katsu et al. [15] investigated the effect of a single low concentration of GS on the release of $K^+$ from

sonicated egg PC vesicles at a temperature of 28°C, well above the gel to liquid–crystalline phase transition of this mixture of phospholipid molecular species. The addition of GS produced an initially rapid efflux of $K^+$ from these liposomes, the rate of which decreased with time; however, the loss of trapped $K^+$ was largely complete by 2 min. The kinetics and extent of $K^+$ loss from egg PC vesicles appeared to be similar to that from *E. coli* cytoplasmic membrane vesicles, but less than that from sheep red blood cells. In a later study, Katsu and coworkers [32] extended these initial studies to include large unilamellar vesicles made from *E. coli* and rat liver mitochondrial membranes lipids, as well as from several different phospholipids or phospholipid mixtures, prepared by reverse-phase evaporation. When the extent of $K^+$ efflux at 5 min was compared, GS at a peptide/phospholipid molar ratio of 1:125 released 80 and 100% of $K^+$ from *E. coli* and rat liver mitochondrial liposomes, respectively, whereas the release of $K^+$ was 80, 100 and 60%, respectively, from egg PC, egg PE/egg PG (10:1), and egg PC/ cholesterol (10:1) liposomes. Thus GS appeared to increase the permeability of lipid bilayers containing either the zwitterionic lipid PE and/or the anionic lipid PG to a greater extent than for those generated from the zwitterionic lipid PC, and the presence of cholesterol appeared to inhibit the GS-induced permeabilization of egg PC liposomes.

Yagi et al. [22] studied the effect of the addition of GS on the leakage of a trapped carboxy fluorescein (CF) dye from DPPC SUVs at 45°C. At lower GS concentrations (DPPC/GS ratio 125:1), a moderate rate of CF leakage was observed, with about half the total CF released in 6 min; dye release was most rapid immediately after peptide addition and decreased steadily with time thereafter. At higher GS concentrations (unspecified), GS induced very rapid and near total release of entrapped dye. These workers suggested that at lower concentrations, GS permeabilized, but did not grossly disrupt, the structure of DPPC vesicles, while at higher concentrations, the peptide probably induced the lysis of these SUVs.

Wu et al. [33] have recently investigated the effect of 12 antimicrobial peptides on planar lipid bilayer membranes generated from a mixture of diphytanoyl PC and diphytanoyl PG (4:1 wt. ratio) separating two aqueous compartments each containing 1 M KCl. The addition of low concentrations of GS (1–2 μg/ml) to one compartment of this system produced transient conductance events due to the periodic movement of $K^+$ across the membrane. The induction of these conductance events required a potential across the membrane of about −80 mV relevant to the side of the membrane from which GS was introduced, and these events were not observed at positive membrane potentials, as expected from the dicationic nature of this peptide. Since these conductance events showed a considerable degree of quantization, but exhibited variable lifetimes (0.5–6.0 s), these workers interpreted their results in terms of GS forming transient $K^+$-conducting channels in this phospholipid bilayer system. Interestingly, GS, at least at the low concentrations tested, did not destabilize the phospholipid membrane at physiologically relevant potentials (−80 to −230 mV), although at −280 mV, membrane breakage was observed only in the peptide-containing system. Thus Wu and coworkers suggested that at the minimal inhibitory concentration, GS acts by permeabilizing rather than grossly disrupting the lipid bilayers of bacterial membranes. These results seem much more compatible with the 'pore' type model than with a 'carpet' type model of the mode of action of this particular antimicrobial peptide. However, it is difficult to construct a plausible molecular model of how GS molecules could arrange themselves in a phospholipid bilayer to form a fairly stable channel with a polar core capable of conducting $K^+$ ions.

We have recently studied the effect on GS on the permeability of large unilamellar phospholipid vesicles in their liquid-crystalline states utilizing a calcein dye leakage assay (unpublished results). At comparable peptide concentrations (phospholipid/peptide ratios of 25:1) and temperatures (37°C), GS was most effective at releasing dye from zwitterionic 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) vesicles (90% release at equilibrium) and least effective at releasing calcein from zwitterionic 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE) vesicles (20% release). This result is expected, since our previous DSC [18] and FTIR [27] and fluorescence spectroscopic (unpublished) studies all showed a stronger interaction of GS or GS analogs with the less ordered DMPC as compared to the more ordered DMPE bilayers at temperatures above
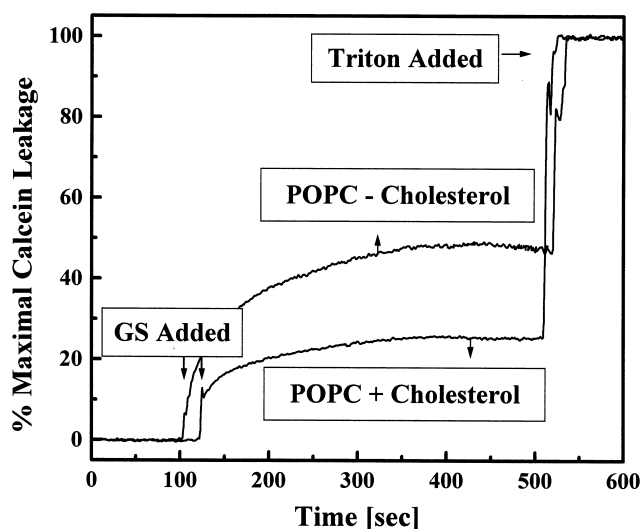
Fig. 10. Plots of the leakage of the entrapped fluorescence dye calcein from large, unilamellar POPC vesicles, containing 0 and 25 mol% cholesterol, as a function of time. At the time indicated by the arrow, GS at a phospholipid/peptide molar ratio of 25:1 was added. The total amount of calcein trapped in the POPC vesicles was determined by adding 1% Triton X-100 to completely lyse the vesicles. Fluorescence was measured at 517 nm using an excitation beam set at 496 nm.

but near their gel to liquid–crystalline phase transition temperature. However, the effect of GS on permeabilizing anionic 1-palmitoyl-2-oleoyl phosphatidylglycerol (POPG) vesicles was intermediate between that observed for the two zwitterionic phospholipids (42% dye release). This result is somewhat unexpected, as our previous biophysical studies [18,20,27] indicate that GS interacts more strongly with anionic DMPG than with either zwitterionic DMPC or DMPE bilayers. Moreover, as discussed earlier, Katsu et al. [32] reported that GS was more effective at releasing $K^+$ from vesicles composed of a mixture of egg PC/egg PG (10:1) than from egg PC alone, suggesting that the presence of an anionic phospholipid potentiates the disruptive action of GS at least in zwitterionic lipid bilayers. We have no explanation for the seemingly anomalous results of GS on the permeability of POPG vesicles, but experiments are ongoing to resolve this issue.

The data presented in Fig. 10 do show, however, that the addition of 25 mol% cholesterol to each of the POPC vesicles significantly reduces calcein leakage. This result confirms and extends the earlier report by Katsu et al. [32], previously discussed, which

indicated that the presence of cholesterol (10 mol%) reduced the permeability of egg PC liposomes to $K^+$. Moreover, the reduction in peptide-induced vesicle permeability by cholesterol is also compatible with our earlier DSC [18] and FTIR [27] and fluorescence spectroscopic (unpublished) studies, which indicated that cholesterol reduces the extent of interaction of GS with DMPC bilayers. We attribute this characteristic effect of cholesterol to a general reduction in the fluidity of liquid–crystalline phospholipid bilayers and to an ordering of the polar/apolar interfacial region of the lipid bilayer by cholesterol.

We have also established that the effectiveness of GS in inducing dye release from various phospholipid vesicles increases with peptide concentration up to some limiting value, as expected. However, our studies on the permeabilization of POPC bilayers at lower peptide concentrations suggest that the dependence of calcein leakage on the amount of GS added is complex, possibly indicating that this peptide may disrupt phospholipid vesicles by more than one mechanism. We have also shown that in POPC vesicles, with and without 25 mol% cholesterol, the ability of GS to induce calcein leakage was only weakly dependent on temperature as long as the phospholipid bilayers remain in their liquid–crystalline states. However, the effectiveness of GS in inducing calcein leakage increased markedly at higher temperatures in POPE bilayers. We attribute this latter effect at least in part to our earlier calorimetric [18] and $^{31}$P-NMR [20] results, indicating that GS does not interact significantly with DMPE and DEPE bilayers until temperatures well above their respective phase transition temperatures are reached, presumably because a sufficient degree of bilayer fluidity is required for peptide penetration. As well, since GS induces inverted cubic phase formation in DMPE bilayers at higher temperatures, the marked increase in the GS-induced permeabilization of DMPE bilayers with temperature may reflect an increasing degree of lamellar phase destabilization by this peptide.

Fluorescence dye leakage experiments from phospholipid unilamellar vesicles have been widely used to study the disruption of the permeability barrier of phospholipid bilayer model membranes by antimicrobial peptides. Although this is a convenient and useful technique for such purposes, it must be em-

ployed with care if valid quantitative and even qualitative results are to be obtained. For example, as mentioned earlier, the rate of dye release may not always be a linear function of peptide concentration, so a range of peptide concentrations should be tested. Moreover, at a constant lipid/peptide ratio, the percent of entrapped dye released may vary significantly with the total concentration of peptide and phospholipid employed. We have also found that the rate and extent of dye release can depend on the size of the vesicles used. The dependence on vesicle size may reflect the influence of the radius of curvature of the vesicles on the degree of binding and the ease of peptide penetration into the outer monolayer of the phospholipid bilayer. If phospholipid and peptide solutions are too concentrated, the scattering of light from the phospholipid/peptide aggregates can also affect the measured fluorescence and peptide–peptide aggregation can occur before addition to the phospholipid vesicles. If, on the other hand, phospholipid and peptide concentrations are too low, kinetic limitations can become important. In this latter regard, the leakage of dye should be followed over a long enough time to ensure that dye release has reached a steady state. We have also found that the pH, ionic strength and the nature of the buffer used can also influence the results obtained for a particular peptide–phospholipid system. Thus, attention to these and other factors is required to generate accurate and reliable information using this technique.

## 4. Interactions of GS with biological membranes

Yonezawa and coworkers [34–36] studied the binding of $^{14}$C-GS and various radioactive GS derivatives to intact cells and to cell wall-less protoplasts of several species of bacteria with different susceptibilities to this antimicrobial peptide. In general, GS absorbed more readily to GS-susceptible bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* than to GS-resistant bacteria such as *E. coli*. In both cases, GS binding increased linearly with increasing peptide concentration up to the minimal inhibitory concentration (MIC) for growth (about 1.0–1.5 μg/ml for *B. subtilis* and *S. aureus*, and 5–10 μg/ml for *E. coli*), and less rapidly thereafter. At the MIC, about $1.3 \times 10^6$ molecules of GS were bound per bacterial cell, enough to cover the cell surface. Interestingly, at the MIC, protoplasts of *B. subtilis* bound about 80% of the GS bound by intact cells, indicating that the majority of added peptide is bound to the bacterial inner membrane and not the extracellular cell wall. The absorption of GS in all cases was shown to increase the permeability of cells and protoplasts and to inhibit amino acid or glucose uptake. These results, plus the generally good correlation between the ability of GS analogs to bind to bacterial cells and their MIC values, led these workers to conclude that the mode of action of GS involves binding to the bacterial inner membrane and the resultant perturbation of inner membrane structure and function.

Kaprel'yants et al. [37], Eremin et al. [38] and Dergunov et al. [39] carried out some of the first biophysical studies of the interaction of GS with bacterial membranes, utilizing protoplasts and isolated inner membranes from *Micrococcus lysodeikticus*. Interestingly, protoplasts required relatively high concentrations of GS for lysis at 37°C, but were increasing more sensitive to peptide-induced lysis at lower temperatures (4–22°C), when both gel and liquid–crystalline lipid domains were present in the inner membrane. However, at sublytic concentrations of peptide, GS increased the permeability of protoplasts for substrates of the respiratory chain and decreased the activity of several respiratory chain enzymes in a peptide-concentration and time-dependent manner. Interestingly, after the addition of GS to protoplast membranes, GS could not be efficiently removed by washing with aqueous salt solutions, but could be largely removed by the addition of an excess of exogenous phospholipid vesicles, indicating that hydrophobic as well as electrostatic interactions must be involved in GS binding. Moreover, if added GS was removed by phospholipid vesicles at early times, respiratory enzyme inhibition could be largely reversed, whereas prolonged incubation (2 h at 30°C) resulted in an irreversible inhibition of some membrane-bound enzymes, even after the peptides was removed, indicating the GS can cause the denaturation of membrane enzymes, either directly or indirectly (see below).

Kaprel'yants and coworkers [37] also studied the location of a fluorescently labeled GS analog ($N,N'$-di[$N^\alpha$-dansylornithyl]GS) by monitoring the wave-

length of maximum fluorescence of this probe in solvents of varying polarity and in *M. lysodeikticus* protoplast membranes. The fluorescence maximum of this analog varied from 525 nm in water to 410 nm in heptane. When bound to membranes, this didansyl derivative of GS exhibited a major maximum at 495 nm and a minor shoulder at 520 nm, indicating localization primarily in an environment of 'intermediate' polarity (polarity between acetone and benzene). This result would seem to be compatible with our DSC [18] and FTIR [27] and fluorescence (unpublished) spectroscopic studies on GS bound to phospholipid bilayer model membranes discussed earlier, which suggested that GS is located at the polar/apolar interfacial region of lipid bilayers, near the glycerol backbone of the lipid molecule. One should note, however, that the didansyl derivative of GS will have a different overall polarity and amphilicity than will GS itself, and that this may affect the results obtained. However, the authors state that this derivative retained an appreciable antimicrobial activity and ability to inhibit membrane-associated enzymes, suggesting that its overall physical properties and mode of action are not too dissimilar to that of the parent peptide.

These workers [37–39] also demonstrated by ESR spectroscopy that the addition of GS to *M. lysodeikticus* protoplast membranes produced an immobilized or motionally restricted lipid population, suggesting that this peptide can induce the formation of boundary or annulus lipid in the liquid–crystalline membrane lipid phase in this system. The addition of GS was also found to decrease the lateral mobility of pyrene in the lipid bilayer and to increase the quenching of the fluorescence of membrane proteins by a quencher localized in the membrane lipid phase. These authors proposed that GS, by preferentially binding membrane lipids through both electrostatic and hydrophobic interactions, causes a partial disruption of interactions between the native boundary lipids and the membrane proteins, thereby inhibiting their function. Although plausible, this hypothesis requires further verification, as GS and other cationic peptides can have direct effects on the conformation and activity of membrane-associated enzymes, as the authors' own work suggests.

The antibiotic activity of ten amphipathic antimicrobial peptides, including GS, in six species of mol-

licutes (cell wall-less bacteria) was recently investigated by Benven and Wroblewski [40]. These workers reported that of all peptides tested, GS was by far the most active. The effect of GS was cidal rather than static, since none of the mollicutes tested was capable of resuming growth when placed on fresh media after treatment with concentrations of peptide above the MIC. When added to growing *Spiroplasma melliferum* cells at their respective MICs, GS produced the most rapid and the greatest reduction in the transmembrane electrical potential across the cell membrane of this organism. Interestingly, however, about 4 min after GS addition, the electrochemical potential difference across the membrane began to recover and returned to normal levels within about 10–15 min, whereas the membrane depolarization produced by the other antimicrobial peptides tested did not recover at all by 20 min. Since GS exerted a strong and permanent cidal effect despite its only transient membrane depolarization, a more complex mechanism of action, possibly involving secondary targets distinct or not from the cell membrane, was suggested. GS, along with melittin, was also the most potent antimicrobial peptide tested in terms of mobility inhibition and cell shape deformation as well.

Hancock and coworkers [33] have recently studied the action of 12 cationic peptides, belonging to all four structural classics of antimicrobial peptides, on the transmembrane electrical potential of an outer membrane barrier-defective strain of *E. coli*. Their general finding was that these peptides differed widely in their ability to depolarize the inner membrane of this organism, and that many of the peptides tested were ineffective at decreasing membrane potential even at concentrations well above their respective MICs, leading those workers to suggest that the cytoplasmic membrane is not the target of some or even most cationic microbial peptides. However, at their respective MICs, GS produced the most rapid and most extensive membrane depolarization of these *E. coli* cells of all the peptides tested and extensive membrane depolarization was already observed at GS concentrations well below the MIC. In this study, however, membrane depolarization by GS appeared to be permanent, rather than transient as in the mollicutes study [40]. Moreover, as discussed earlier, GS was also one of the most potent

antimicrobial peptides tested in terms of its ability to produce conductance events (channels) in planar phospholipid bilayer membranes. In addition, Katsu et al. [15] had shown earlier a good correlation between the MICs of various GS analogs and their ability to increase the permeability of the inner membrane to $K^+$ in both Gram-positive and Gram-negative bacteria. Therefore, these studies all support the hypothesis that the primary target of GS is the lipid bilayer of bacterial membranes.

## 5. Summary and conclusions

Although a substantial amount of biophysical data is available concerning the interactions of GS with lipid model and with biological membranes, much remains to be done. In particular, the discrepant results of certain DSC studies of the effects of GS on phospholipid thermotropic phase behavior need to be resolved. Moreover, there is also considerable disagreement in the literature over the affinity for and the location of GS in phospholipid bilayer membranes and the effect of bilayer-associated peptide on phospholipid orientation and dynamics. In regard to the former, accurate determinations of the partition coefficients of GS between water and a variety of phospholipid bilayer systems in their biologically relevant liquid-crystalline states should be a priority. As well, a careful determination of the thermodynamics of GS binding to a variety of phospholipid bilayer systems would be most useful. We are currently attempting to provide such information by compiling a complete thermodynamic profile of GS binding to various large unilamellar phospholipid vesicles. Specifically, we are utilizing isothermal titration calorimetry to determine the binding constant, binding stoichiometry, and the enthalpy and entropy of GS binding to phospholipid bilayers whose chemical composition and physical properties are systematically varied. In this way, considerable insight into the still incompletely characterized lipid specificity of GS/phospholipid bilayer interactions can be obtained. Finally, additional studies of the actual mechanism of action of GS on model phospholipid bilayer and on biological membranes are clearly required. It is our view that until such additional data are available, the construction of a detailed model of the mode of action of GS on model and biological membranes is premature. Moreover, we suspect that GS may have different modes of action on different phospholipid bilayers, and that the interaction of this peptide with a given phospholipid bilayer may vary with the amount of peptide present and with the physical state of the target membrane.

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