Fourier Transform Infrared Spectroscopic Studies of the Interaction of the Antimicrobial Peptide Gramicidin S with Lipid Micelles and with Lipid Monolayer and Bilayer Membranes†

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ABSTRACT: We have utilized Fourier transform infrared spectroscopy to study the interaction of the antimicrobial peptide gramicidin S (GS) with lipid micelles and with lipid monolayer and bilayer membranes as a function of temperature and of the phase state of the lipid. Since the conformation of GS does not change under the experimental conditions employed in this study, we could utilize the dependence of the frequency of the amide I band of the central β-sheet region of this peptide on the polarity and hydrogen-bonding potential of its environment to probe GS interaction with and location in these lipid model membrane systems. We find that the GS is completely or partially excluded from the gel states of all of the lipid bilayers examined in this study but strongly partitions into lipid micelles, monolayers, or bilayers in the liquid-crystalline state. Moreover, in general, the penetration of GS into zwitterionic and uncharged lipid bilayer coincides closely with the gel to liquid-crystalline phase transition of the lipid. However, GS begins to penetrate into the gel-state bilayers of anionic phospholipids prior to the actual chain-melting phase transition, while in cationic lipid bilayers, GS does not partition strongly into the liquid-crystalline bilayer until temperatures well above the chain-melting phase transition are reached. In the liquid-crystalline state, the polarity of the environment of GS indicates that this peptide is located primarily at the polar/apolar interfacial region of the bilayer near the glycerol backbone region of the lipid molecule. However, the depth of GS penetration into this interfacial region can vary somewhat depending on the structure and charge of the lipid molecule. In general, GS associates most strongly with and penetrates most deeply into more disordered bilayers with a negative surface charge, although the detailed chemical structure of the lipid molecule and physical organization of the lipid aggregate (micelle versus monolayer versus bilayer) also have minor effects on these processes.

Gramicidin S (GS)[1] is a cyclic decapeptide [cyclo-(Val-Orn-Leu-D-Phe-Pro)$_2$] first isolated from *Bacillus brevis* and is one of a series of antimicrobial peptides produced by this microorganism (see refs *1* and *2*). This peptide exhibits appreciable antibiotic activity against a broad spectrum of both Gram-negative and Gram-positive bacteria as well as against several pathogenic fungi (*1*−*4*). Unfortunately, GS is rather nonspecific in its actions and exhibits appreciable hemolytic as well as antimicrobial activity. The therapeutic utilization of GS has therefore been limited to topical applications. A major goal of our current studies on the interaction of GS with model and biological membranes is to provide the fundamental knowledge of the mechanism of action of this peptide on lipid bilayers that is required to design GS analogues with enhanced activity for bacterial membranes and diminished activity against the plasma membranes of human and animal cells.

Considerable evidence exists that the primary target of GS is the lipid bilayer of cell surface membranes and that this

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[1] Abbreviations: GS, gramicidin S; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin (diphosphatidylglycerol); PS, phosphatidylserine; PA, phosphatidic acid; SpM, sphingomyelin; DLPC, 1,2-dilauroylphosphatidylcholine; DMPC, 1,2-dimyristoylphosphatidylcholine; DPPC, 1,2-dipalmitoylphosphatidylcholine; DLPE, 1,2-dilauroylphosphatidylethanolamine; DMPE, 1,2-dimyristoylphosphatidylethanolamine; DEPE, 1,2-dielaidoylphosphatidylethanolamine; DTPC, 1,2-tetradecylphosphatidylcholine; DMPG, 1,2-dipalmitoylphosphatidylglycerol; TMCL, 1,2-tetramyristoylcardiolipin; MGDG, monoglucosyldiacylglycerol; DM-TAP, dimyristoyl-3-(*N*,*N*,*N*-trimethyl)propane; DGDG, diglucosyldiacylglycerol; DE-MGDG, 1,2-dielaidoyl monoglucosyldiacylglycerol; DE-DGDG, 1,2-dielaidoyl diglucosyldiacylglycerol; MeOH, methanol; MeOD, methanol-d_1; EtOH, ethanol; EtOD, ethanol-d_1; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; TFA, trifluoroacetate; FTIR, Fourier transform infrared; IRRAS, infrared reflection−absorption spectroscopy; NMR, nuclear magnetic resonance; ESR, electron spin resonance; DSC, differential scanning calorimetry; T_m, gel/liquid-crystalline phase transition temperature.

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peptide kills cells by destroying the structural integrity of the lipid bilayer by inducing the formation of pores or other localized defects (see refs *2*−*5*). However, only a limited number of studies of the interaction of this peptide with lipid bilayer model membranes have been published to date (for a recent review, see ref *5*). Moreover, the conclusions reached from these studies do not always agree with one another. Pache et al. (*6*) reported that, even at a low lipid/peptide molar ratio (10:1), GS had almost no effect on the gel to liquid-crystalline phase transition temperature, enthalpy, or cooperativity of DPPC multilamellar vesicles, nor was the organization the DPPC bilayer significantly perturbed by the presence of the peptide as monitored by NMR or ESR spectroscopy. The only significant effect of GS addition observed was a decrease in the DPPC P−O stretching frequency as monitored by IR spectroscopy. Pache and coworkers thus concluded that GS is bound to the surface of DPPC bilayers and interacts only with the lipid polar headgroups. A similar conclusion was reached by Datema et al. (*7*) based on their observations of the very small reduction of the temperature and cooperativity of the main phase transition of DPPC observed by DSC even at low lipid/peptide molar ratios (6:1), the insensitivity of the motional characteristics of the GS molecule to the DPPC gel to liquid-crystalline phase transition, and the ability of GS to induce an isotropic component in the ^{31}P-NMR spectrum at higher temperatures. However, based on the generally appreciable hydrophobicity of the GS molecule, the sensitivity of the lateral diffusion coefficient of this peptide to the host bilayer chain-melting phase transition, and the ability of cholesterol to induce lateral aggregation of GS in DPPC and particularly in DMPC bilayers, Wu et al. (*8*) suggested that GS must penetrate at least partially into the hydrophobic core of the host lipid bilayer. This conclusion was later supported by Zidovetzki et al. (*9*), who demonstrated by ^{2}H-NMR spectroscopy that GS alters the orientational order parameter profile of the hydrocarbon chains of DMPC, at least at low lipid/protein molar ratios (5.5:1), and by ^{31}P-NMR spectroscopy, which suggested that the structure of the bilayer is abolished at even lower lipid/peptide molar ratios (2.7:1). Thus these early studies, which were done exclusively on zwitterionic PC systems, failed to resolve either the issue of the location of GS in lipid model membranes or the nature of its interactions with the host lipid bilayer.

We have recently studied the effect of GS on the thermotropic phase behavior of a variety of synthetic phospholipids using primarily ^{31}P-NMR spectroscopy supplemented by DSC and X-ray diffraction (*10*). We found that, at physiologically relevant concentrations of GS (lipid/protein molar ratios of 25:1), GS does not affect the lamellar phase preference of the zwitterionic lipids PC and SpM nor of the anionic lipids CL and PS, even at high temperatures. However, GS was found to potentiate inverted cubic phase formation in zwitterionic PE and, to a lesser extent, in anionic PG dispersions as well as in total polar lipid extracts from the glycolipid-based membranes of *Acholeplasma laidlawii* and from the phospholipid-based membranes of *Escherichia coli*. Moreover, the ability of GS to induce nonlamellar phase formation was found to increase with the intrinsic nonlamellar phase-forming propensity of the PE molecular species studied. We suggested that the ability of GS to induce localized regions of high curvature stress in the lipid bilayers of biological membranes may be relevant to the mechanism by which this peptide disrupts cell membranes.

We have also studied the effects of GS on the thermotropic phase behavior of large multilamellar vesicles of DMPC, DMPE, and DMPG by high-sensitivity DSC (*11*). We found that the effect of GS on the lamellar gel to liquid-crystalline phase transition of these phospholipids varies markedly with the structure and charge of their polar headgroups. Specifically, the presence of even large quantities of GS has essentially no effect on the main phase transition of zwitterionic DMPE vesicles, even after repeating cycling through the phase transition unless these vesicles are exposed to high temperatures, after which a small reduction in the temperature, enthalpy, and cooperativity of the gel to liquid-crystalline phase transitions is observed. Similarly, even large amounts of GS produce modest decreases in the temperature, enthalpy, and cooperativity of the main phase transition of DMPC vesicles, although the pretransition is abolished at low peptide concentrations. However, exposure to high temperatures is not required for these effects of GS on DMPC bilayers to be manifested. In contrast, GS has a much greater effect on the thermotropic phase behavior of anionic DMPG vesicles, substantially reducing the temperature, enthalpy, and cooperativity of the main phase transition at higher peptide concentrations and abolishing the pretransition at lower peptide concentrations as compared to DMPC. Moreover, the relatively larger effects of GS on the thermotropic phase behavior of DMPG vesicles are also manifest without cycling through the phase transition or exposure to high temperatures. These 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. As well, 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.

Our recent work has thus confirmed earlier studies that GS does exhibit an appreciable polar headgroup specificity when interacting with various glycerophospholipid and sphingophospholipid bilayers. We are therefore continuing to study the nature of the interactions of GS with lipid bilayer model membranes that contain a wide range of polar headgroups. This research is intended to provide basic information on the sensitivity of various membrane lipid classes to the action of GS and on how these are affected by variations in the structure of the peptide molecule. Because the lipid compositions of bacterial and eucaryotic cell membranes are quite different and because antimicrobial and hemolytic activities can be at least partially dissociated in GS analogues (*4*), such information could potentially be used in the design of more therapeutically useful GS derivatives.

The three-dimensional structure of the GS molecule is shown in Figure 1. In this minimum energy conformation, the two tripeptide sequences Val-Orn-Leu form an antipar-

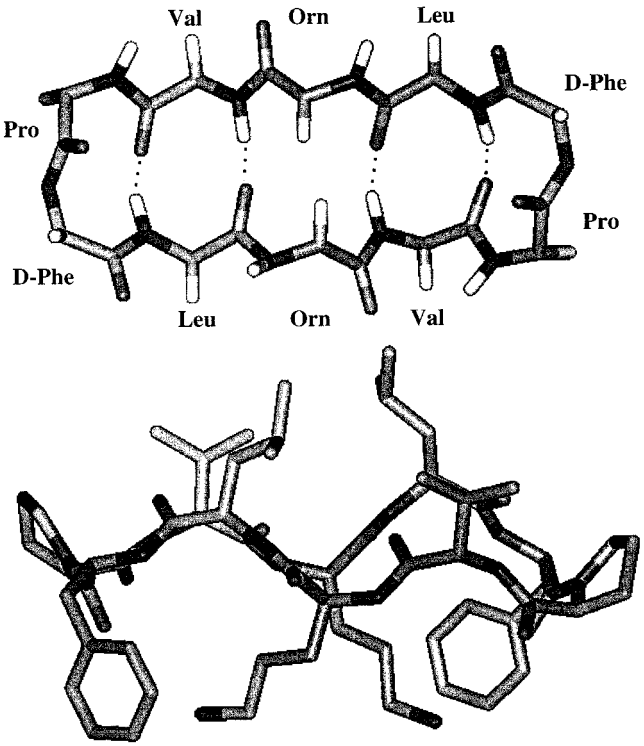

FIGURE 1: Structure and conformation of GS. (Upper panel) Top view of the backbone structure of GS indicating the positions of the β-sheet interstrand hydrogen bonds. (Lower panel) Side view of GS indicting the disposition of the hydrophobic (top) and basic (bottom) residues relative to the peptide ring.

allel β-sheet terminated on each side by a type II′ β-turn formed by the D-Phe-Pro sequences. Four intramolecular hydrogen bonds, involving the four amide protons and the four carbonyl groups of the Val and Leu residues, stabilize this rather rigid structure. Note that the GS molecule is amphiphilic with the two somewhat polar and positively charged Orn side chains projecting from one side of this molecule and the four hydrophobic Val and Leu side chains projecting from the other side. This amphiphilic character of the GS, with two positively charged amino acid residues on one face of the peptide molecule and four hydrophobic residues on the other, is required for maximal antimicrobial activity, as is the overall β-sheet conformation (see refs *1* and *2*).

A large number of studies have established that the characteristic conformation of the GS molecule is maintained in water, in protic and aprotic solvents of widely varying polarity, and in detergent micelles and in phospholipid bilayers, even at high temperatures and in the presence of protein-denaturing agents (see refs *1* and *2*). We take advantage of this fact here to relate changes in the frequency of the amide I absorption band of the β-sheet region of the GS molecule to the polarity and hydrogen-bonding potential of the environment of the peptide molecule in the gel and liquid-crystalline phases of various lipid assemblies, since such frequency changes should not arise from alterations in the secondary or tertiary structure of the GS molecule. This somewhat unique approach has permitted us to determine the polarity and hydrogen-bonding potential of the environment of the GS molecule when interacting with various lipid aggregates and thus its degree of association and probable location in these various model membrane systems.

MATERIALS AND METHODS

The phospholipids used in this study were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and were used without further purification. The glycolipids were purified from the polar lipid extracts of elaidic acid−homogeneous *Acholeplasma laidlawii* B using previously published procedures (*12*). Gramicidin S was obtained from Sigma (St. Louis, MO) and purified by previously published, high-performance liquid chromatographic methods (*3, 4*). For most of the samples used in this study, the peptide was twice lyophilized from 10 mM hydrochloric acid to ensure complete removal of the trifluoroacetate. The latter gives rise to a strong infrared absorption band ($\cong$1673 cm^{-1}) that can overlap the amide I absorption bands of the peptide (*13*). All deuterated solvents were obtained from Aldrich (Milwaukee, WI).

The preparation of samples for FTIR spectroscopy proceeded as follows. Dried samples were examined either as lyophylized powders or as dry films. The former were usually samples lyophylized from aqueous solutions of the peptide, and the latter were films prepared from solutions of the peptide in volatile organic solvents such as methanol and ethanol. Spectra of the lyophylized powders were recorded with the aid of a Spectra Tech diffuse reflectance accessory, whereas those of films were recorded using standard transmittance techniques as applied to films dried onto the surfaces of CaF_2 windows. Liquid-based samples were prepared as follows. Samples containing 1−2 mg of peptide were dissolved in the appropriate solvent, and the solution or paste was then squeezed between the CaF_2 windows of a heatable liquid cell (equipped with a Teflon spacer) to form a 25-μm film. Once mounted in the sample holder of the instrument, the sample temperature could be controlled (between $\sim$20° and 90 °C) by an external, computer-controlled circulating water bath. With lipid-containing samples, the peptide and GS were co-dissolved in methanol to obtain a lipid:peptide ratio of 25:1, and the solvent was removed with a stream of nitrogen and subsequent overnight evacuation. Next, the mixture was hydrated by the addition of 75−100 μL of a D_2O buffer containing 50 mM phosphate and 100 mM NaCl (pH 7.4), followed by vigorous vortexing at temperatures well above the gel/liquid-crystalline phase transition of the lipid. This procedure produced a paste, which was used as described above. With such samples, infrared spectra were recorded with a Digilab FTS-40 infrared spectrometer (Digilab, Cambridge, MA) using data acquisition parameters comparable to those described by Mantsch et al. (*14*).

For monolayer film samples spread at the air/water interface, infrared spectra were recorded using the trough and optical setup described by Flach et al. (*15*). For this series of IRRAS experiments, stock solutions of lipid and GS were prepared by dissolving the samples in a chloroform/methanol solution (2:1, v:v) at a concentration of $\cong$2 mg/mL, and appropriate amounts of the stock solutions were mixed to prepare lipid/peptide solutions at a molar ratio of 10:1. The monolayer film was prepared by spreading 4−5 μL of the lipid- and peptide-containing solution on an aqueous subphase having a surface area of $\cong$83 cm^2. The subphase consisted of a buffer containing 10 mM Tris, 100 mM NaCl, and 100 μM EGTA in D_2O (pD 7). The initial surface

pressure values of these GS−phospholipid films before compression was 1−5 mN/m. After an initial relaxation period of 1 h, the film was intermittently compressed over a 3−4-h period to reach the desired surface pressure. At this final pressure, the film was allowed to relax for at least 1 h prior to IR data collection. Data were acquired with a Digilab FTS 40A spectrometer equipped with an MCT detector. These experiments were conducted using unpolarized infrared radiation and an angle of incidence (i.e., the angle between the incoming beam and surface normal) of 35°. Typically, 1024 scans were acquired at 4 cm^{-1} resolution, apodized with a triangular function, and Fourier transformed with one level of zero-filling to yield spectra encoded at 2 cm^{-1} intervals. IRRAS spectra are presented as reflectance absorbance (RA) vs wavenumber (cm^{-1}) plots. RA is defined at $-\log(R/R^F)$, where R is the reflectivity of the film-covered surface and R^F is the reflectivity of the film-free surface. All infrared spectra obtained were analyzed off-line with Galatic Instruments (Salem, NH) Grams/32 software and other computer programs obtained from the National Research Council of Canada and from Microcal Software Inc. (Northampton, MA).

RESULTS

One should note that two different FTIR spectroscopic techniques have been utilized in this study—conventional transmission FTIR spectroscopy and IRRAS. The former technique will simultaneously monitor the amide I absorption bands of all the GS molecules in the system, both those associated with the lipid assemblies and those dissolved in the aqueous phase. In contrast, the IRRAS technique monitors primarily those GS molecules associated with the phospholipid monolayer at the air−water interface or in close proximity thereto and not those dissolved in the bulk aqueous subphase at a distance from the monolayer. One should also note that, in the transmission FTIR experiments, the volume of the aqueous phase relative to that of the lipid micelles or bilayer vesicles is much less than is the ratio of the volume of the aqueous phase to that of the lipid monolayers utilized in the IRRAS technique.

Transmission FTIR Spectroscopic Characterization of GS in Dry Form or Dissolved in Various Solvents. To confirm the extensive earlier work establishing that the secondary and tertiary structure of the GS molecule remains constant in different environments (see refs *1* and *2*), we studied solid GS and GS dissolved in solvents of different polarity and hydrogen-bonding potential by transmission FTIR spectroscopy. The contours of the amide I regions of these GS spectra are presented in Figure 2. In all cases, the amide I band consists solely of a relatively sharp major band located at a lower frequency (about 1630−1650 cm^{-1}) and a broader minor band located at a higher frequency (about 1665−1675 cm^{-1}), the latter partly obscured by the sharp band at 1673 cm^{-1} arising from the TFA counterion in the case of the TFA salt of GS (*13*). The lower frequency band, which accounts for at least 70% of the total integrated intensity of the amide I absorption, is characteristic of peptides and proteins that adopt an antiparallel β-sheet conformation (see refs *16*−*18*), as expected, confirming earlier IR results (*19*−*21*). The higher frequency band, which accounts for 30% or less of the total integrated amide I intensity in the HCl salt of GS, we ascribe to the type II′ β-turns components of the GS structure contributed by the proline and D-phenylalanine residues, which would not be involved in the formation of internal hydrogen bonds (see ref *22*), again as in previous studies (*19*−*21*). Although this higher frequency band is partly obscured in the TFA salt form of GS, this is not a problem here since we utilize only the major lower frequency amide I absorption band in the rest of this study. Note also that the amide I frequency of this band is the same for both TFA and HCl forms of GS when dissolved in D_2O (Table 1) or in several phospholipid bilayer systems (data not shown). As well, our earlier NMR spectroscopic, X-ray diffraction, and DSC studies have shown that the TFA and HCl salts of GS interact similarly with various phospholipid bilayers (*10*, *11*). Finally, note that amide I bands corresponding to other peptide conformations (helical, random, etc.) are absent when the peptide is dissolved in various solvents or when associated with lipid aggregates, even at high temperatures.

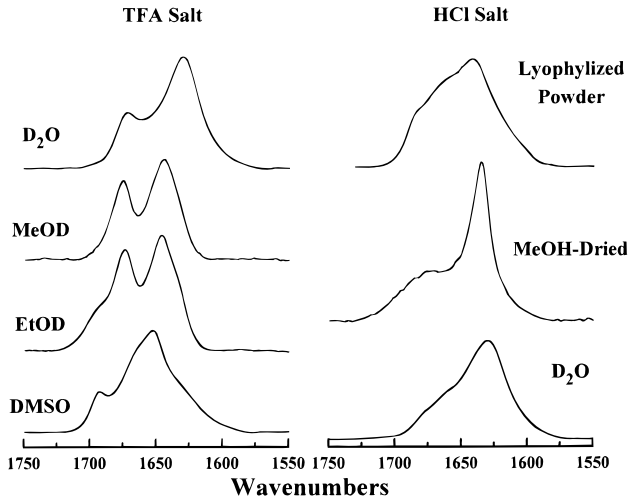

FIGURE 2: FTIR absorbance spectra showing the amide I bands of the trifluoroacetyl (left) and hydrochloride (right) salts of gramicidin S.

Table 1: Amide I Band Maxima of Gramicidin S Dissolved in or Dried from Various Solvents

sample	band maxima (cm^{-1})	comments
HCl salt	1639	lyophylized powder
HCl salt	1636	MeOH dried film
HCl salt	1629	D_2O solution
TFA salt	1629	D_2O solution
TFA salt	1643	MeOD solution
TFA salt	1646	EtOD solution
TFA salt	1652, 1662[a]	DMSO solution

[a] High frequency shoulder on the 1652 cm^{-1} band.

Although the overall contour of the amide I absorption band is preserved in the various environments examined here, a careful inspection of Figure 2 reveals that the frequency of the major lower frequency component can vary appreciably, as detailed in Table 1. When the HCl salt of GS is dried from the protic solvents methanol or water, the major amide I component is centered at a frequency of 1635−1639 cm^{-1}. These values compare favorably with the results of earlier studies, which reported a frequency value for this band of 1637 cm^{-1} in similar systems (*20*, *21*). However, when dissolved in D_2O, the frequency of the major amide absorption band of GS decreases to 1629 cm^{-1}, doubtlessly due to

hydrogen−deuterium exchange of the internal peptide bonds that are being monitored here (see refs *23*−*25*). Of key importance for this work, however, is the effect of dissolution in a series of solvents of different polarity and hydrogen-bonding potential. Thus, as shown in Table 1, the frequency of the major amide I band of the TFA salt of GS increases progressively from 1629 to 1643 to 1646 cm^{-1} when the peptide is dissolved in D_2O, MeOD, and EtOH, respectively. This result indicates that, in a series of protic solvents, the frequency of this band increases as the polarity of the solvent decreases. Moreover, when dissolved in the aprotic but relatively polar solvent DMSO, the frequency of the major amide I band of GS is shifted to even higher frequencies (1652−1663 cm^{-1}). Since the characteristic conformation of GS has been reported to be the same in all of the solvent systems utilized here (see refs *1* and *2*), we ascribe these changes in the frequency of the amide I band of GS to changes in the polarity and hydrogen-bonding potential of its environment. This ascription is certainly reasonable since the amide I band frequency of various methyl-N-alkyl and C-alkyl amides has also been shown to vary markedly depending on the polarity and hydrogen-bonding potential of their environment (see ref *26*). Specifically, the amide I frequency in these compounds varies from about 1620 to 1660 cm^{-1} in a series of protic solvents of progressively decreasing polarity and from about 1650 to 1690 cm^{-1} in a series of aprotic solvents of decreasing polarity. In the subsequent sections of this paper, we use the relationship between amide 1 band frequency and the polarity and hydrogen-bonding potential of the environment to probe the interactions of GS with lipid micelles and with lipid monolayer and bilayer membranes.

Transmission FTIR Spectroscopic Characterization of GS Associated with Lipid Micelles and Bilayers. (a) Dependence of GS Amide I Frequency on Temperature and Lipid Phase State. The polarity and hydrogen-bonding potential of the environment of the GS molecule when reconstituted with a variety of lipid micellar or bilayer systems in excess D_2O was studied by transmission FTIR spectroscopy as a function of temperature, so that the nature of the interaction of GS with both the gel and the liquid-crystalline phases of the host lipid could be examined. In all of these studies a physiologically relevant lipid−GS molar ratio of 25:1 was utilized. A typical example of the results obtained are illustrated in Figure 3, where stacked plots of the amide I band of the GS molecule and the lipid carbonyl stretching band are shown as a function of temperature for the DMPA/GS binary mixture. The GS amide I band is centered at a frequency of 1631 cm^{-1} at temperatures between 20 and 48 °C and is almost temperature invariant over this range of temperatures. Similarly, at temperatures above 52 °C, the GS amide I band is centered at a much higher frequency of 1647 cm^{-1} and is again also almost temperature invariant. Note that the shift in the peptide amide I frequency of about 16 cm^{-1} near 50 °C is also accompanied by an increase in the width and a decrease in the overall frequency of the carbonyl stretching mode of the host DMPA bilayer (Figure 3) and by an increase in the frequency of the methylene symmetric stretching vibration of the DMPA bilayer hydrocarbon chains (data not presented), FTIR spectroscopic changes associated with gel to liquid-crystalline phospholipid phase transitions (see refs *27*−*29*). Note also that GS dissolved in D_2O alone

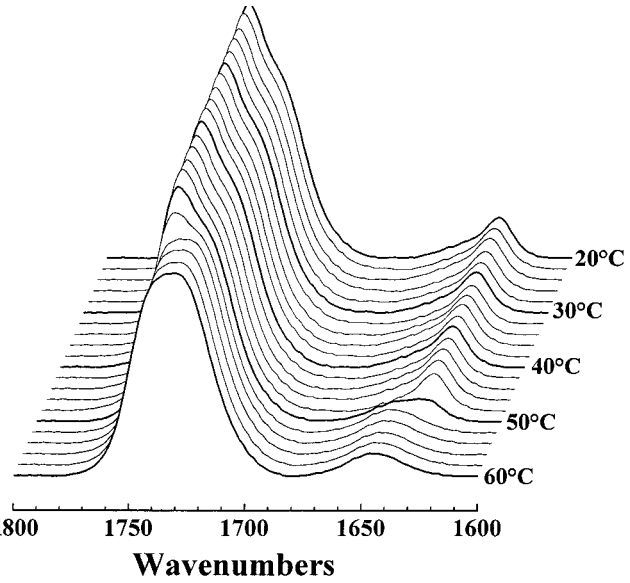

FIGURE 3: FTIR absorbance spectra showing the phospholipid C═O stretching and peptide amide I absorptions exhibited by a DMPA/GS (25:1) mixture. The data are presented as a stacked plot showing the temperature-dependent changes in the lipid C═O stretching and peptide amide I band between 20 and 60 °C. Spectra were acquired in intervals of 2 °C, and those highlighted by the bold lines were acquired at the temperatures indicated.

exhibits an amide I frequency of 1629 cm^{-1} that does not change significantly over the temperature range examined here. Since the conformation of GS does not change with temperature or with the physical state of the host lipid bilayer (see refs *1*, *2*, and the present study), these results indicate that GS resides in a relatively polar environment with considerable hydrogen-bonding potential in the presence of gel-state DMPA bilayers but in a relatively less polar environment of reduced hydrogen-bonding potential in the presence of liquid-crystalline DMPA bilayers. As well, since the amide I band frequency of GS in the presence of gel-state bilayers is near that of the D_2O solvent, we interpret this observation to mean that the GS molecules in this case are largely excluded from the DMPA bilayer proper, associating instead with the lipid polar headgroups at the bilayer surface or existing largely free in solution. However, the frequency of the GS amide I band associated with bilayers above the phase transition temperature suggests an environment of considerably lower but intermediate polarity, such as might be associated with the polar/apolar interfacial region of the phospholipid bilayer (*30*). At any rate, a major shift of the amide I band of this peptide to higher frequencies is generally associated with the hydrocarbon chain-melting phase transition of essentially every lipid micellar or bilayer system examined in this study (see Table 2).

The thermotropic behavior of most of the lipid/GS mixtures examined in this study generally resembles that of the DLPE/GS system illustrated in Figure 4B, in that the shift in the amide I frequency of the peptide coincides approximately in temperature with the shifts in the carbonyl and methylene stretching vibrations of the lipid matrix, such that the GS molecule appears to insert into the fluid bilayer concomitant with the lipid gel to liquid-crystalline phase transition. In fact, this behavior is characteristic of all of the zwitterionic phospholipid and nonionic glycolipid species examined in this study. However, for the three anionic

Table 2: Amide I Band Maxima of Gramicidin S in D_2O Dispersed Lipid Bilayers

lipid host	surface charge	band maxima (cm^{-1}) gel phase[a]	band maxima (cm^{-1}) fluid phase[b]
DLPC	±	1638[c]	1646
DMPC	±	1638[c]	1645
DPPC	±	1636[c]	1644
diphytanoylPC	±	*d*	1643
DTPC	±	1636[c]	1643
1-O−palmitoyllysoPC	±	1634[e]	1642 [f]
1-O−stearoyllysoPC	±	1635[e]	1643 [f]
DLPE	±	1629	1639
DMPE	±	1629	1639
DEPE	±	1629	1639
DMPA	−	1631	1647
DMPG	−	1636[c]	1648
TMCL	−	1628	1642
P−O-ethyl-DMPC	+	1632[c]	1641
DM-TAP	+	1629	1629[g]
DE-MGDG	nonionic	1630	1640
DE-DGDG	nonionic	1630	1641
Acholeplasma 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 and 1641−1645 cm^{-1}. [d] Lipid exists in a fluidlike phase at all temperatures examined. [e] Interdigitated coagel phase: summation of bands centered near 1630 and 1642 cm^{-1}. [f] Fluid phase is micellar. [g] Insertion only starts at drastically increased temperatures near 75 °C (T_m plus 40 °C); a maximum amide I frequency of 1640 cm^{-1} is observed at 90 °C.

phospholipids examined (DMPA, DMPG, and TMCL), the shift in the GS amide I frequency on heating begins to occur at significantly lower temperatures than the shifts in either the lipid carbonyl or methylene stretching bands, as illustrated for the TMCL/GS system in Figure 4A. Moreover, in these anionic phospholipid systems, we also note that the shift in the shape and frequency of the lipid carbonyl stretching vibration begins at a lower temperature and occurs over a wider temperature range than does the actual melting of the lipid hydrocarbon chains, as monitored by the shift in the symmetric methylene stretching frequency. Since such a temperature offset between the lipid carbonyl and methylene frequency shifts is not observed with these anionic phospholipids in the absence of peptide, we interpret these results to indicate that GS molecules begin to insert into the interfacial region of these negatively charged phospholipid bilayers prior to the actual gel to liquid-crystalline phase transition. Moreover, this insertion appears to destabilize these gel-state bilayers since our earlier calorimetric studies (*10*, *11*) and our present FTIR spectroscopic investigation both indicate that, at these relatively high peptide concentrations, the addition of GS lowers the gel to liquid-crystalline phase transition temperature of the host lipid matrix. In contrast, in the positively charged P−O−ethyl-DMPC/GS mixture, the shift in the frequency of the amide I band of GS does not begin until just above the gel to liquid-crystalline phase transition temperature (see Figure 4C), and this shift in frequency continues at temperatures well above those at which the lipid phase transition is complete. Moreover, in the case of the cationic lipid DM-TAP/GS mixture, no shift in the GS amide I frequency at all occurs until temperatures well above the gel to liquid-crystalline phase transition are reached. Even at 75 °C an amide I band component near 1629 cm^{-1} persists, indicating that a portion of GS molecules are excluded even from this presumably rather fluid lipid bilayer (data not presented). These results suggest that, in these latter systems, the peptide molecules cannot insert into the liquid-crystalline bilayer until a sufficiently high degree of temperature-induced disorder has been obtained. We have reported similar behavior, although less extreme, for GS/DMPE mixtures previously (*10*, *11*).

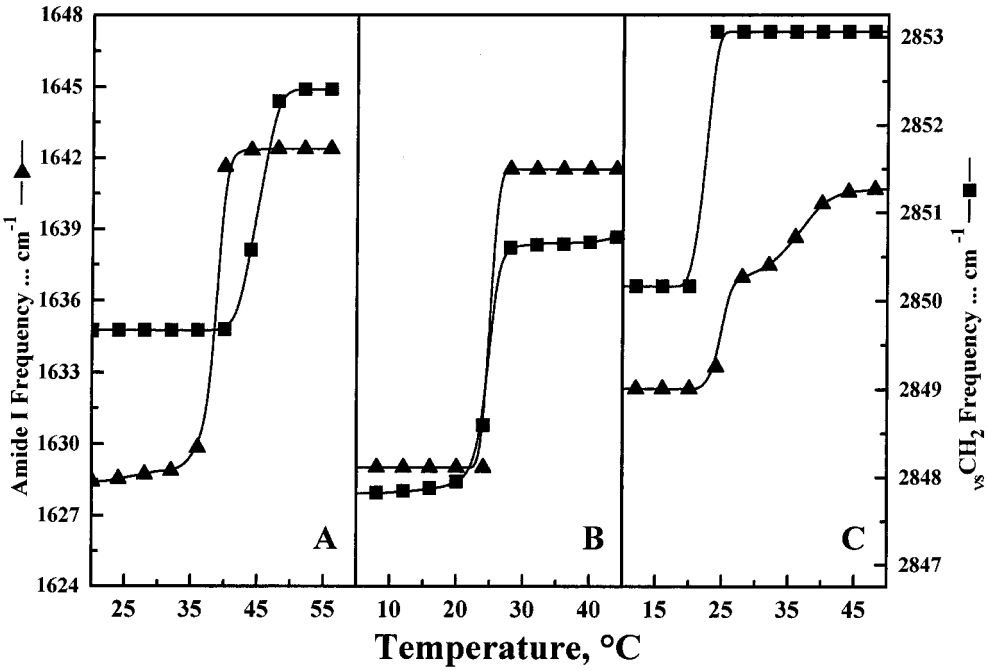

FIGURE 4: Temperature-dependent changes in the peak frequencies of the phospholipid hydrocarbon chain CH_2 symmetric stretching (■) and peptide amide I (▲) absorption bands of mixtures of GS with TMCL (A), DLPE (B), and P−O−ethyl-DMPC bilayers (C). The pattern of amide I shifts relative to CH_2 stretching shifts are representative of those observed with anionic bilayers (A), zwitterionic and nonionic bilayers (B), and cationic bilayers (C).

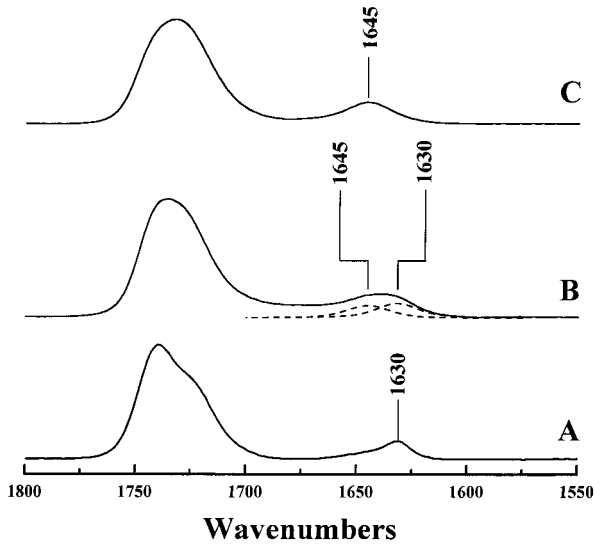

FIGURE 5: FTIR absorbance spectra showing the phospholipid C=O stretching and peptide amide I absorptions of GS dispersed in gel (A and B) and liquid-crystalline (C) bilayers. The amide I absorption band of spectrum A (DMPA/GS mixture) typifies our observations of bilayers that form highly ordered gel phases whereas that shown in spectrum B (DMPC/GS mixture) typifies those of bilayers that form relatively disordered gel phases.

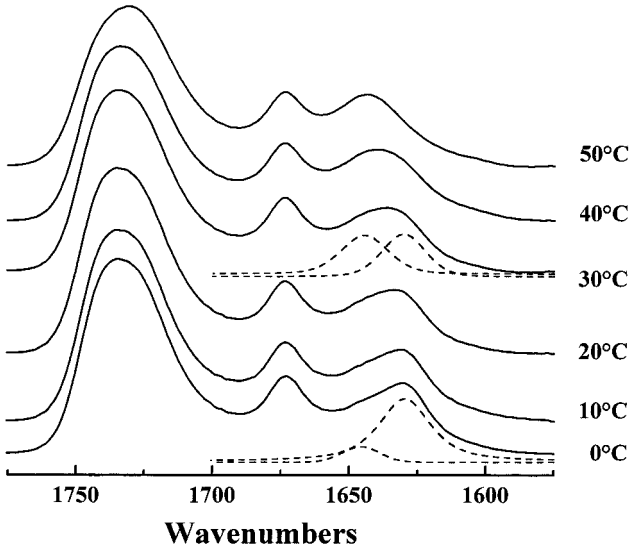

FIGURE 6: FTIR absorbance spectra illustrating the temperature-dependent changes in the amide I absorption band of GS (TFA salt) dispersed in DPPC bilayers. The solid lines show the spectra acquired, and the dashed lines show estimates of the relative sizes of the amide I components centered near 1630 and 1645 cm^{-1}. Note that the relative intensity of the high-frequency component decreases upon cooling.

(b) GS Amide I Frequency in Gel State Bilayers. The data presented in Table 2 indicate that there is considerable variation in the frequency of the GS amide I band in the gel states of the various lipid bilayer systems examined. This variation is largely accounted for by the fact that there may be either one or two components to this band, depending on the particular lipid system examined. For example, in the DMPA/GS system illustrated in Figure 5A, the amide I band in the gel state appears to consist of a single component centered near 1630 cm^{-1}. This component is observed in the gel states of all of the lipid micelles or bilayers examined here, and in many systems (all the PEs, DMPA, TMCL, MGDG, and DGDG), this is the only amide I band component present. However, in the gel state of the DMPC/GS mixture illustrated in Figure 5B, there are two components in the peptide amide I band: one centered near 1630 cm^{-1} and another centered at a much higher frequency of 1645 cm^{-1}. Note that the lower frequency component disappears or is at least markedly reduced above the gel/liquid-crystalline phase transition but that the higher frequency component persists and is the only component present in the liquid-crystalline state. Similar behavior is noted for all the PCs examined, for DMPG, for the *A. laidlawii* B total membrane lipids, and for the lyso PCs studied, the latter of which form fully interdigitated bilayer phases in the gel state. Moreover, as shown in Figure 6, as the temperature of gel-state DPPC bilayers is decreased, the relative contribution of the higher frequency components of the GS amide I band decreases while that of the lower frequency component increases. Following the rationale discussed above, we interpret these results to mean that, in some gel-state bilayers, two populations of GS molecules exist, one partially inserted into the bilayer and one largely excluded from the bilayer, and that further ordering of the gel-state bilayer by a reduction in temperature can result in the progressive exclusion of a greater proportion of the peptide molecules from the lipid matrix.

In addition to determining the effects of variations in lipid polar headgroup structure on the polarity and hydrogen-bonding potential of the environment of GS molecules in the presence of various gel-state bilayers, we also explored the effects of variations in the length and structure of the lipid hydrocarbon chains in gel-state bilayers of the zwitterionic lipid classes PC and PE (see Table 2). Although the structure of the lipid polar headgroup is important in determining whether the GS molecule can penetrate into the gel-state bilayer, the length of the hydrocarbon chain or the presence of a single trans-double bond in both chains had little, if any, effect on the association or lack thereof of the peptide molecule with the gel-state PE bilayer. However, a small tendency for the peptide amide I frequency to decrease with increasing hydrocarbon chain length in the PC series is noted. As well, the removal of one fatty acyl chain to produce a lysoPC, which forms a fully interdigitated coagel phase below the chain-melting phase transition temperature, also does not affect the spectroscopic behavior of the peptide molecule greatly, although an apparently smaller degree of association of GS with these lysoPCs as opposed to DPPC matrixes was observed. Similarly, when the diacylphospholipid DMPC is compared with its diether counterpart DTPC, a somewhat lower overall frequency of the GS amide I band is noted, again suggesting a lower degree of penetration of the peptide molecule into the gel state of the latter compound. However, the magnitude of these effects are small as compared to those produced by variations in polar headgroup structure.

(*c) GS Amide I Frequency in Liquid-Crystalline Bilayers or Micelles*. As noted previously, the overall GS amide I frequency always shifts upward by 7−16 cm^{-1} in the general region of the chain-melting phase transition temperature of the lipid matrix with which it is associated, and the lower frequency component near 1630 cm^{-1}, in particular, shifts upward by 10−18 cm^{-1}. Thus, as shown in Table 2, the peptide amide I frequency varies considerably in the liquid-

crystalline state despite the fact that all the values presented in Table 2 were obtained at roughly comparable reduced temperatures above the gel to liquid-crystalline phase transition temperature of the peptide-containing lipid matrix. The highest frequencies were exhibited by the two anionic phospholipids DMPG and DMPA (1647−1648 cm^{-1}), intermediate frequencies were exhibited by the zwitterionic PCs and the *A. laidlawii* B total membrane lipids (1643−1646 cm^{-1}), and the lowest frequencies were exhibited by the zwitterionic PEs, the anionic DTCL, the positively charged P−O−ethyl-DMPC and DM-TAP, and the neutral glycolipids MGDG and DGDG (1640−1642 cm^{-1}). As a general rule, those lipid bilayer systems that exhibit the lowest overall frequency of the GS amide I band in the gel state (i.e., those lipid bilayers exhibiting only a single-component amide I band near 1630 cm^{-1}) also exhibit the lowest amide I band frequency in the liquid-crystalline state. The two exceptions to this rule are the anionic phospholipid DMPA−peptide mixture, which shows a low GS amide I frequency in gel state but a high frequency in liquid-crystalline bilayers, and the positively charged phospholipid P−O−ethyl-DMPC peptide mixture, which shows a relatively high peptide amide I band frequency in gel state but a relatively low frequency in liquid-crystalline bilayers. Note that the absolute frequencies of the GS amide I band in these liquid-crystalline lipid matrixes vary considerably, ranging from 1640 to 1648 cm^{-1}, indicating that the polarity and/or hydrogen-bonding potential of the environment in which the peptide molecule is located in these various lipid systems also varies, ranging from an apparent polarity on the protic solvent scale presented in Table 1 from somewhat more polar than methanol to somewhat less polar then ethanol. However, the absence of a significant amide I band frequency near 1630 cm^{-1} indicates that relatively few, if any, GS molecules are present in the bulk aqueous phase or even absorbed to the polar headgroups at the surface of these liquid-crystalline micellar or bilayer systems.

The effects of variations in the length and structure of the hydrocarbon chains on the polarity and hydrogen-bonding potential of the environment of GS in liquid-crystalline zwitterionic PC and PE bilayers were also examined (see Table 2). Increasing the length of the linear saturated hydrocarbon chain in the PC series (DLPC, DMPC, DPPC) resulted in a small reduction of peptide amide I frequency, as did the introduction of multiple methyl branches (compare DPPC and diphytanoyl PC), as noted previously for gel-state PC bilayers. However, in the PE series, no changes in GS amide I frequency with hydrocarbon chain length or degree of trans-unsaturation were observed, just as in gel-state bilayers. The removal of one hydrocarbon chain as in lysoPPC, which forms normal micelles above its phase transition temperature, results in a lower peptide amide I frequency, as does replacement of the ester groups linking the hydrocarbon chain to the glycerol backbone (DMPC) with either linkages (DTPC). These latter two effects were also noted in these systems in their gel states.

(d) IRRAS Studies of GS Amide I Frequencies in Phospholipid Monolayers. To focus exclusively on GS molecules associated with the lipid matrix, IRRAS experiments on DPPC/GS and DPPG/GS mixed monolayers spread at the air/water interface were carried out. Under the conditions at which these experiments were performed, these phospholipid

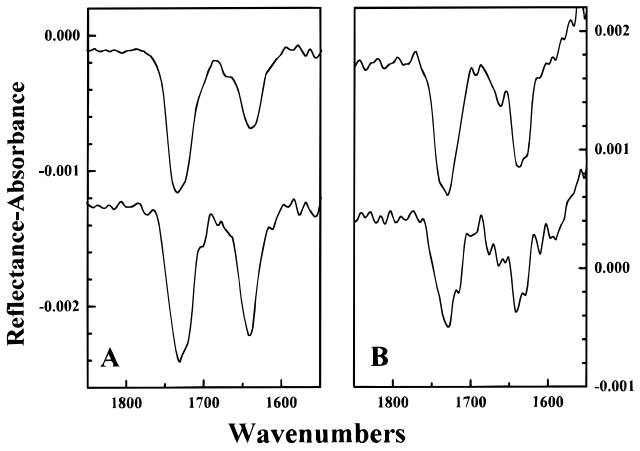

FIGURE 7: IRRAS spectra showing the C═O stretching of the phospholipid and amide I regions of GS in DPPG (A) and DPPC (B) monolayers spread at the air/water interface. The top spectrum in each panel shows data acquired at a surface pressure of 16 mN/m, and the bottom spectrum shows data acquired at a surface pressure of 6 mN/m. To facilitate comparison of the two data sets, the intensities of the bands in the top spectrum have been normalized relative to the integrated intensity of the phospholipid C═O stretching band near 1730 cm^{-1} of the bottom spectrum.

monolayers exist in the liquid-condensed phase at higher pressure (16 mN/m), corresponding most closely to the gel phase of lipid bilayers, and in the liquid-expanded phase, which most closely corresponds to the liquid-crystalline phase of lipid bilayers, at the lower pressure (6 mN/m). IRRAS spectra of the DPPG/GS monolayer at the two surface pressures are shown in Figure 7A. A more intense GS amide I absorption is observed at the lower pressure, suggesting that a portion of the peptide is squeezed out of the monolayer at higher surface pressures. In both cases, however, the amide I band is centered at approximately 1643 cm^{-1}, and a major component located near 1630 cm^{-1} is absent. In contrast, the predominant amide I band for pure GS monolayers is observed at 1630 cm^{-1} over a surface pressure range of 4−15 mN/m (data not shown). These results indicate that the GS molecules actually associated with the liquid-condensed and liquid-expanded DPPG monolayers do indeed reside in an environment of relatively lower polarity and/or hydrogen-bonding potential than do the peptide molecules dissolved in or residing at the surface of the aqueous phase and that the amide I absorption band near 1630 cm^{-1} observed in the transmission FTIR experiments does indeed arise from GS molecules not associated with the lipid matrix. As well, the increased peptide amide I absorption associated with the liquid-expanded as compared to the liquid-condensed DPPG monolayers is also compatible with our transmission experiments on DMPG bilayers, which indicate a higher proportion of GS molecules residing in the liquid-crystalline state as opposed to the gel-state matrix of this particular class of phospholipids.

The comparable IRRAS spectra of the DPPC/GS monolayer at two surface pressures is shown in Figure 7B. In contrast to the results obtained with the anionic DPPG/GS monolayer, two peptide amide I bands are observed here, suggesting the existence of two populations of GS molecules associated with the zwitterionic DPPC monolayer. The major amide I band centered at 1640−1643 cm^{-1} presumably arises from GS molecules that have inserted into the lipid monolayer and the minor band at 1630 cm^{-1} to GS molecules

associated with the monolayer but exposed to the aqueous subphase. Note that the frequency of the major band appears to increase in going from higher to lower surface pressure, suggesting that the GS may penetrate more deeply into the DPPC monolayers in the liquid-expanded state as compared to the liquid-condensed state. Note also that the ratio of the intensity of the lower frequency band to that of the higher frequency band increases at higher pressure, suggesting that less GS is present in liquid-condensed state as compared to liquid-expanded monolayers. Surface pressure−molecular area isotherms of the DPPC/GS monolayers support this conclusion since at the monolayer collapse pressure the apparent area per phospholipid molecule (about 0.44 nm^2) approaches that of the DPPC monolayer in the absence of peptide (data not presented). In contrast, in the DPPG/GS monolayers, the apparent area per phospholipid molecule at the collapse pressure (about 0.70 nm^2) is considerably larger than that observed for the pure DPPG monolayer, indicating that some GS remains inserted into the DPPG monolayer even at high surface pressures.

DISCUSSION

Our finding that the frequency of the amide I band arising from the β-sheet portion of GS is dependent on the polarity and hydrogen-bonding potential of its environment coupled with the fact that the conformation of the GS molecule is invariant under our experimental conditions (see refs *1* and *2*) has permitted us to monitor the relative sizes of the populations of GS associated with the lipid micelles and bilayers and with the aqueous phase under various conditions. In gel-phase bilayers of some lipid systems, GS exhibits only a single amide I band with a frequency near 1630 cm^{-1}, which is also characteristic of GS dissolved in D_2O, and little or no absorption at the higher frequencies characteristic of less polar environments. In the gel phases of other lipid bilayer systems, two amide I bands are present, one near 1630 cm^{-1} and a second at a higher frequency (1642−1648 cm^{-1}), the proportion of these two bands varying with the nature of the lipid being investigated (see below) and with the temperature. Thus, in the former systems, GS seems largely excluded from these gel-phase bilayers, while in the later systems some association of GS with gel-state bilayers is occurring, although a substantial portion of this peptide remains in the aqueous phase. Interestingly, the amide I frequencies of GS molecules that are associated with some gel-state bilayers are generally rather similar to the amide I frequency observed in the liquid-crystalline states of these same bilayers, suggesting a comparable location in the bilayer in both cases.

In all of the phospholipid bilayer systems examined, the overall amide I frequency shifts upward in frequency as the GS/phospholipid mixture is heated from temperatures below to temperatures above the lipid gel to liquid-crystalline phase transition temperature. In all cases, this upward shift in frequency results entirely from the loss of the low frequency band at about 1630 cm^{-1} and its replacement by a higher frequency band centered at 1639−1648 cm^{-1}, the latter value depending on the lipid system being examined. This result indicates that, in the liquid-crystalline state, the vast majority of the GS reside within the lipid micelle or lipid bilayer. In fact, in the fluid lipid systems, we can estimate that the lipid/water partition coefficient of GS must be at least 10^3 in favor of the lipid, otherwise a measurable amide I band with a frequency at about 1630 cm^{-1} would have been detectable given the larger volume of the aqueous phase relative to the lipid phase. However, we cannot assess quantitatively how the lipid/water partition coefficient of GS varies with the nature of the host lipid micelle or bilayer because of the strong associations of the peptide with these fluid systems, although the results of the IRRAS experiment clearly indicate that the partition coefficient must be somewhat higher for the anionic lipid DMPG than it is for the zwitterionic lipid DMPC in the monolayer/water system.

In various liquid-crystalline bilayers or micelles, the frequency of the GS amide I band varies from about 1639 to 1648 cm^{-1}, frequencies clearly much higher than exhibited by GS dissolved in D_2O and ranging in frequency from somewhat lower than GS dissolved in MeOD to somewhat higher than GS dissolved in EtOD. Although we could not determine the amide I frequency of GS in very nonpolar organic solvents such as liquid hydrocarbons due to solubility limitations, the very low polarity of such solvents and their complete lack of hydrogen-bonding potential would suggest that the amide I frequency would be at least as high as that of GS in DMSO (1652−1662 cm^{-1}), a relatively polar aprotic solvent with limited hydrogen-bond-forming ability. Therefore, the intermediate amide I frequency values observed in liquid-crystalline bilayers suggest the GS is located primarily in the interfacial region of these phospholipid micelles or bilayers rather than at the level of the polar headgroups or hydrocarbon core of the bilayer. Although the GS amide I frequencies observed in the IRRAS experiments with DMPC and DMPG liquid-expanded monolayers are somewhat less than observed for GS in DMPC and DMPG liquid-crystalline bilayers, these frequencies still reflect an environment of intermediate polarity. Interestingly, a location of GS near the glycerol backbone of the phospholipid molecules is also compatible with our previous DSC study of GS/phospholipid bilayer systems and with most of the results from previous studies of these systems (see refs *10* and *11*).

As mentioned above, the frequency of the GS amide I band in various liquid-crystalline micelles or bilayers varies considerably, from about 1639 to 1648 cm^{-1}. In principle, this variation could be due to a difference in the average depth of penetration of the GS molecules into the polar/apolar interfaces of these fluid lipid systems and/or to an intrinsic difference in the polarity of this interface. Since very little hard data are available about the absolute or comparative polarity of the polar/apolar interfacial region of different phospholipid bilayer systems, we attempted to estimate this parameter in a semiquantitative way by monitoring the apparent polarity of the lipid carbonyl groups of the fatty acyl chains by determining the weighted average of the frequencies of the lipid carbonyl stretching frequencies in the absence of peptide. Since the frequency of this band is known to decrease considerably with the polarity of the environment, in particular with the number of hydrogen bonds formed between the carbonyl group and the solvent molecules (*31*−*33*), the average carbonyl band frequency should provide at least a crude estimate of the relative intrinsic polarity or hydrogen-bonding potential of the polar/apolar interfaces of the various liquid-crystalline bilayers examined here. Although the average carbonyl stretching

frequencies exhibit some variation in these pure lipid systems, there appears to be no general correlation between the apparent polarity of the polar/apolar interface of the liquid-crystalline bilayer system and the frequency of the GS amide I band arising from GS molecules associated with such systems. We therefore tentatively conclude that the variations in the frequency of the GS amide I band in various liquid-crystalline bilayers primarily reflect the extent of penetration of the GS molecule into these bilayer interfaces rather than intrinsic differences in the polarity of these interfaces themselves. Clearly, however, this conclusion will have to be validated by more direct physical methods in future studies.

We believe that most of our results can be rationalized by assuming that the two major physical properties of the host lipid micelle or bilayer that determine the degree of association or penetration of GS molecules with these systems are lipid aggregate fluidity and surface charge. A change in relative fluidity or order can explain the observation that GS molecules are excluded or partly excluded from the more highly ordered gel-state bilayers generally, while partitioning strongly into the interfacial region of the less highly ordered liquid-crystalline bilayers or lipid micelles. An increase in the degree of order in gel-state DPPC bilayers with a decrease in temperature can also explain the progressive exclusion of GS molecules from this lipid system at lower temperatures. Moreover, GS is completely excluded from those lipids that form more highly ordered gel phases (the PEs, DMPA, MGDG, DGDG) as manifested in the relatively high gel to liquid-crystalline phase transition temperatures of these lipids (see ref *34*) but shows some penetration into the less highly ordered gel phases formed by other lipids (the PCs, lysoPCs, DMPG, *A. laidlawii* B total membrane lipids). Finally, the smaller degree of penetration of GS into the diether phospholipid DTPC in comparison to its diacyl analogue DMPC in the gel state can also be explained by the higher degree of order in the gel-state bilayers of the former system.

A change in the relative fluidity or order of lipid systems in the liquid-crystalline state can also explain many of the results obtained with micelles and bilayers. Thus, the amide I frequency of GS is generally higher in those lipids (the PCs, lysoPCs, DMPG, *A. laidlawii* total membrane lipids) that form relatively disordered lipid micelles or bilayers as compared to those lipids forming relatively more highly ordered liquid-crystalline bilayers (the PEs, MGDG, DGDG) (*34*−*37*). Similarly, just as in the gel-state systems, GS exhibits a smaller extent of penetration into the more highly ordered DTPC as compared to the less highly ordered DMPC liquid-crystalline bilayers (*38, 39*). Similar results have been reported by us in previous papers (*10, 11*) and are also supported by our finding that the presence of cholesterol, which orders liquid-crystalline bilayers, also reduces the interaction of GS with PC and PG vesicles (*40*). However, the relative fluidity of liquid-crystalline lipid systems may not explain all of the results obtained in this study, even when lipid surface charge is not a factor. For example, the apparently greater extent of GS penetration into PC bilayers with shorter hydrocarbon chains, in the gel as well as the liquid-crystalline state, does not appear to be explainable by relative fluidity arguments since evidence exists that, at comparable reduced temperatures, at least the hydrocarbon chains are overall more highly ordered in the shorter chain liquid-crystalline bilayers (*41, 42*). Similarly, the overall smaller extent of penetration of GS into LPPC micelles as opposed to DPPC bilayers is also not explainable on this basis since the micellar system exhibits a greater degree of hydrocarbon chain disorder than does the bilayer system at comparable reduced temperatures (*43*). Thus, the detailed chemical structure of the molecules making up the host lipid system and the type of structure formed by these lipids appear to also have some effect on GS−lipid interactions.

The charge of the lipid molecule also has a considerable effect on the association of GS molecules with the lipid bilayer. As documented earlier, GS begins to penetrate gel-state lipid bilayers formed from anionic lipids before the gel to liquid-crystalline phase transition actually occurs, whereas GS molecules do not begin to penetrate cationic lipid bilayer until the phase transition is nearly complete and may not partition strongly into such bilayers until temperatures well above the lipid hydrocarbon chain-melting phase transition are reached. Also, the greatest extent of GS penetration into liquid-crystalline systems is observed in the anionic DMPA and DMPG bilayers, and the presence of PG in the *A. laidlawii* total membrane lipids, which consist mostly of MGDG and DGDG, results in greater GS penetration. However, the GS amide I frequency is comparatively low in TMCL bilayers above their phase transition temperature, perhaps due to a higher degree of hydrocarbon chain order in this system. Similarly, the absence of significant GS association with DMPA and TMCL bilayers below their phase transition temperatures is probably due to the highly ordered gel state formed by these anionic lipids. Thus, although the dicationic GS molecules generally have a tendency to interact more strongly with anionic phospholipids as we reported previously (*10, 11*), it seems clear that this tendency can be modulated by relative lipid order or fluidity, and thus both factors must be considered together in order to rationalize the present results. The higher affinity of GS for anionic as opposed to zwitterionic phospholipids is also documented by the present IRRAS results comparing DMPC and DMPG mixed monolayers. That attractive electrostatic interactions between the positively charged GS molecule and negatively charged phospholipid polar headgroups exist and are important in vivo is supported by the previously observed requirement for two positively charged amino acid residues on the GS molecule for maximal antimicrobial activity and by the fact that the addition of Ca^{2+}, which binds to anionic phospholipid polar headgroups and stabilizes the bilayer structure, inhibits the antimicrobial action of GS (see refs *1* and *2*). However, the present results indicate that the presence of a negatively charged polar headgroup per se is neither a necessary nor a sufficient condition for GS penetration into fluid lipid bilayers, although it does appear to potentiate this process.

Finally, our previous DSC, ^{31}P NMR, and calcein leakage studies (*10, 11, 40*) have shown that, in addition to lipid bilayer fluidity and surface charge density, the propensity of a lipid bilayer to undergo conversion to a reversed nonlamellar (inverted cubic or hexagonal) phase is also a major factor that determines the susceptibility of bilayer vesicles to disruption by GS. Interestingly, in the present study, the two lipids classes (PE and MGDG) that are most susceptible to conversion to reversed nonlamellar phases and to bilayer disruption by GS actually show a relatively weak

interaction with this peptide as monitored by FTIR spectroscopy. In particular, GS is essentially excluded from the highly ordered gel-state bilayers formed by PE and MGDG, and GS penetrates less deeply into the relatively ordered liquid-crystalline bilayers formed by these two lipid classes than into many other lipids. Evidently GS does not need to penetrate deeply into liquid-crystalline bilayers to induce monolayer curvature stress and thus induce inverted nonlamellar phase formation. Interestingly, however, GS can induce various liquid-crystalline PE bilayers to form an inverted cubic phase only after prior exposure to relatively higher temperature in the presence of peptide, which probably increases the depth of penetration of GS into these systems.

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