THE EFFECTS OF VISCOSITY ON GRAMICIDIN TRYPTOPHAN ROTATIONAL MOTION

SUZANNE F. SCARLATA
Division of Digestive Diseases, College of Medicine, Cornell University Medical College, New York, New York 10021

ABSTRACT The rotational amplitude of gramicidin tryptophans was investigated as a function of temperature and viscosity in a variety of solvents using fluorescence spectroscopy. In 80% glycerol-ethanol, gramicidin behavior was similar to that of alpha helical globular proteins. In dioleoyl-phosphatidylcholine (DOPC) and egg-phosphatidylcholine bilayers, the rotational amplitude of the tryptophans remained constant from 5° to 40°C due to the large number of tryptophans participating in intermolecular aromatic ring stacking. In gel phase dimyristoyl-phosphatidylcholine (DMPC), the tryptophan rotations likewise do not respond to temperature and viscosity changes, presumably because of a combination of Trp 9 and 15 stacking and the high viscosity of the membrane. In fluid phase DMPC, stacking becomes disrupted as the temperature increases causing the change in tryptophan amplitude with temperature to be greater than allowed by the membrane. In *n*-octylglucoside micelles, ring interactions are also broken with heat. We conclude that membrane viscosity regulates both inter- and intramolecular gramicidin interactions but not in a straightforward manner.

INTRODUCTION

Gramicidins are small peptides having the general formula

HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D
-Val-L-Val-D-Val-L-Trp-D-Leu-L
-*X*-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2$-CH_2OH

in which X = Trp, Phe, or Tyr at the approximate ratio of 7:1:2 and where the NH_2-terminal Val is sometimes replaced by Ile. The peptides are produced by *Bacillus brevis* during sporulation. The function of gramicidin is believed to be the regulation of gene expression through binding to the alpha subunit of RNA polymerase or to superhelical DNA or a combination of these mechanisms (Fisher and Blumenthal, 1982; Bohg and Ristow, 1986). In reconstituted membranes, gramicidin forms ion-specific channels and has become a model system for understanding transmembrane ion transport (for a review see Andersen, 1984).

The gramicidin channel is an N–N terminal dimer of beta (6.3) helices, similar to the structure first proposed by Urry (1971). In this conformation, the tryptophan side chains point away from the channel cavity and are relatively close to the membrane surface. The tryptophans appear to help stabilize the channel conformation through aromatic ring stacking between tryptophans 9 and 15 (Jones et al., 1986). In organic solvents or anhydrous lipid systems, gramicidin forms a cylindrical intertwined antiparallel double helix whose crystal structure has recently been solved (Wallace and Ravikumar, 1988; Langs, 1988). In this conformation, the tryptophan side chains are more evenly distributed along the outside of the backbone. Upon hydration of the lipids, the peptide unwinds and takes on the channel conformation. If the acyl chain length exceeds 16 carbon atoms (the length of the gramicidin monomer), aggregation, promoted by intermolecular ring stacking, will occur, and at significant protein-to-lipid ratios (e.g., 1:80 in erythrocyte membranes), the complex becomes hexagonal (see Killian and de Kruijff, 1988 and references therein). Tryptophan residues are necessary for the hexagonal phase to form (Killian et al., 1985). Ring stacking has also been found to be important in stabilizing the lamellar structures formed by gramicidin-lysophosphatidycholine dispersions (Spisni et al., 1983). However, the strength of ring interactions has been disputed (Aslanian et al., 1986).

The fluorescence emission properties of the tryptophan residues of gramicidin have been previously characterized (Cavatorta et al., 1982; Masotti et al., 1986). Here, we will determine the rotational behavior of these residues in micelles, as well as dioleoyl-phosphatidylcholine (DOPC), eggPC, and dimyristoyl-phosphatidylcholine (DMPC) vesicles through the fluorescence anisotropy as analyzed in terms of the thermal coefficient of the frictional resistance to fluorophore rotation or **b**. Rotational motion is described by the fluorescence anisotropy $\langle A \rangle$ which is determined by the amount of depolarization of exciting light with respect to its value in the absence of rotation $\langle A_o \rangle$ through the Perrin equation:

$$\langle A_o \rangle / \langle A \rangle - 1 = RT \cdot \langle \tau \rangle / (\eta \cdot V),$$

where R is the gas constant, T the absolute temperature, $\langle\tau\rangle$ the average fluorescence lifetime, η the viscosity, and V the hydrodynamic rotational volume.

The thermal coefficient of the viscosity, **b**, is based on an empirical expansion that relates the viscosity at any temperature (T) to the viscosity at any arbitrary temperature (η_o, T_o) in a given temperature range through the following (Weber et al., 1984):

$$\eta = \eta_o \exp[-\mathbf{b}(T - T_o)].$$

For convenience, we let T_o to 273°K so that $(T - T_o)$ becomes the centigrade temperature, t. The above equation can be substituted into the Perrin equation and rearranged to yield

$$Y = \ln(\langle A_o\rangle/\langle A\rangle - 1) - \ln(RT \cdot \langle\tau\rangle/V) = q + \mathbf{b} \cdot t,$$

where the constant q is equal to $-\ln(\eta_o)$ at 0°C. Y is then the negative logarithm of the restriction to fluorophore rotation caused by its surroundings. By measuring Y as a function of temperature, the thermal coefficient of the viscosity is obtained. While the slopes of a Perrin plot are related to the increases in rotational volume that occur as the temperature is raised or the viscosity lowered, **b** focuses on the changes that occur in the environment around the fluorophore rather than the fluorophore itself.

Weber et al. (1984) originally described **b** for simple fluorophores in isotropic solvents and found that the thermal coefficient, as measured microscopically by fluorescence anisotropy, is the same as the macroscopic **b** as measured by flow viscometry. The magnitude of **b** appears to be proportional to the strength of the intermolecular forces in the solvent.

Small peptides and proteins in a solvent viscous enough to dampen slow motions (80% glycerol-20% buffer), always display at least two **b** values (Scarlata et al., 1984; Rholam et al., 1984): One at high viscosities equal to that of the external solvent and another at lower viscosities whose magnitude is distinctive of the individual protein. From surveying many proteins it followed that at very low temperatures or high viscosities, the rotational amplitude of the fluorophore is very small and any increase is due to the relief of restrictional forces from the external solvent. However, at a certain temperature called the critical temperature, the amplitude becomes large enough such that the fluorophore starts to couple its motion with neighboring peptides and any increase in rotational amplitude is then determined by the solvent of **b** as modified by the peptide. The magnitude of **b** was found to depend on the flexibility of the peptide environment with higher values of **b** relating to more rigid environments. The transition from one **b** value to the next occurs at viscosities of around one poise.

The thermal coefficient of the viscosity of *n*-octylglucoside micelles, and DOPC and DMPC bilayers and multilayers has been recently characterized using various anthroyloxy fatty acid probes (Scarlata, S. F., manuscript submitted for publication). While micelles displayed a thermal coefficient close to butanol, the **b** values of the membranes were much higher than what would be expected for the corresponding isotropic solvents. This behavior was interpreted to be due to extended dispersion interactions along the lipid chains. In DOPC, **b** was found to be constant with membrane depth and in either the x-y or y-z planes of the bilayer. From 5° to 40°C, DMPC displays three **b** values: one for the gel, one for the liquid crystal, and another for the transition region that spanned approximately eight degrees. When protein was added to DMPC bilayers and the motions of anthroyloxy fatty acid viewed, only a single **b** value was observed. In proteoliposomes the lack of a clear phase transition has been observed and it is thought to be due to the disordering of the liquid crystal phase by the protein (see Lee, 1983).

Here we will determine whether membranes, since they have internal viscosities close to a poise (Shinitzky and Barenholz, 1978, and references therein), can couple and decouple peptide motions through changes in the fluidity analogous to globular proteins in 80% glycerol-water. Specifically, the rotational properties of gramicidin tryptophan rings will be investigated both to understand how membrane fluidity may effect its coupled motions and also to learn more about the conformational dynamics of this peptide. We note that this analysis will give averaged information about the rotational behavior of all the emitting species and will thus provide general information on the peptide as a whole and not the individual fluorophores. Also, since many membrane proteins have external aromatic groups, the results of this study may provide information about how bilayers may control peptide dynamics.

MATERIALS AND METHODS

Gramicidin A′ and *n*-octylglucoside were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and used as received. DOPC and DMPC were from Avanti Polar Lipids, Inc. (Birmingham, AL). 11-Anthroyloxyundecanoic acid and 12-anthroyloxy stearic acid were from Molecular Probes, Inc. (Junction City, OR). Thin-layer chromatography showed the probes to be pure. Gold-labeled spectral grade glycerol was from Aldrich Chemical Co. (Milwaukee, WI). *N*-Acetyltryptophanamide was from Sigma Chemical Co. (St. Louis, MO).

Gramicidin was incorporated into bilayers at various molar ratios by mixing the protein and lipid in chloroform or chloroform and ethanol, drying under nitrogen and under vacuum, adding 50 mM Hepes, 160 mM KCl, pH 7 buffer to hydrate and then extensive vortexing. The dispersions were sonicated under nitrogen with a sonicator (model W225; Heat Systems-Ultrasonics, Inc., Farmingdale, NY) at 37°C for a maximum of 30 min or until the mixture was not cloudy. The solution was then spun at 39,000 *g* for 30 min to remove titanium particles and multilamellar vesicles. The same procedure was to incorporate anthroyloxy fatty acid probes. Anthroyloxy probe to lipid ratios were always 0.5% mol/mol. All vesicles were used within 3 days of preparation.

Peptide incorporation was checked by comparing the sedimentation of reconstituted bilayers to that of unlabeled bilayers and free gramicidin by spinning the samples at 39,000 *g* for 18 h in a 25–85% continuous glycerol gradient. Incorporation was also verified by the inability of iodide ions to quench the fluorescence of the proteoliposomes as compared with free

gramicidin in ethanol. Gramicidin concentration was estimated using an extinction coefficient of 22,500 $cm^{-1}mol^{-1}$ (Cavatorta et al., 1982). Lipid concentrations were determined by phosphate analysis.

Circular dichroism (CD) was measured on a Jobin-Yvon Mark 5 spectrophotometer. Corrected fluorescence spectra and anisotropy measurements were taken on a Greg-PC (I.S.S. Inc., Champaign, IL) equipped with Glan-Thompson polarizers. All anisotropy data were the average of a minimum of four independent experiments. Gramicidin was excited at either 280 or 305 nm and emission intensity was collected at 345 nm. Sample intensities were corrected for background scattering using unlabeled vesicles and matching the scattering intensities at 500 nm. Excitation and emission wavelengths of 381 and 460 nm were used for anthroyloxy fluorescence. Phase-modulation lifetimes were taken on an SLM4800 spectrofluorometer (SLM Instruments, Inc., Urbana, IL) using a modulation frequency of 30 MHz. Average lifetime values were used in the Y calculations. The preliminary heterogeneity analysis and time-resolved anisotropy measurements were made on the home-built system utilizing a Nd-YAG dye laser for excitation. Temperatures were controlled by circulating baths.

RESULTS

Studies in 80% Glycerol-20% Ethanol

Gramicidin tryptophan side chain motions were first characterized in 80% glycerol-ethanol (wt/wt) to compare its behavior to globular proteins. The absorption and emission spectra matched those in pure ethanol. Fluorescence anisotropy measurements were taken using an excitation wavelength of 305 nm to avoid homotransfer between the tryptophan residues (Weber, 1960). The Perrin plot was determined by varying the temperature from −35° to 25°C and it showed the protein to have a limiting anisotropy of 0.273 and a molecular volume of 32 ml/mol. Using an anisotropy of 0.4 for collinear dipoles, the angle between the absorption and emission dipoles of gramicidin is calculated to be 27°. This value is similar to free tryptophan and to globular proteins that contain a single tryptophan (Scarlata, 1984). Since energy transfer between tryptophan residues causes depolarization, the high limiting anisotropy indicates that transfer does not significantly occur in the frozen system exciting on the red edge of the absorption band. The low volume corresponds to a very limited motion of the tryptophan side chains.

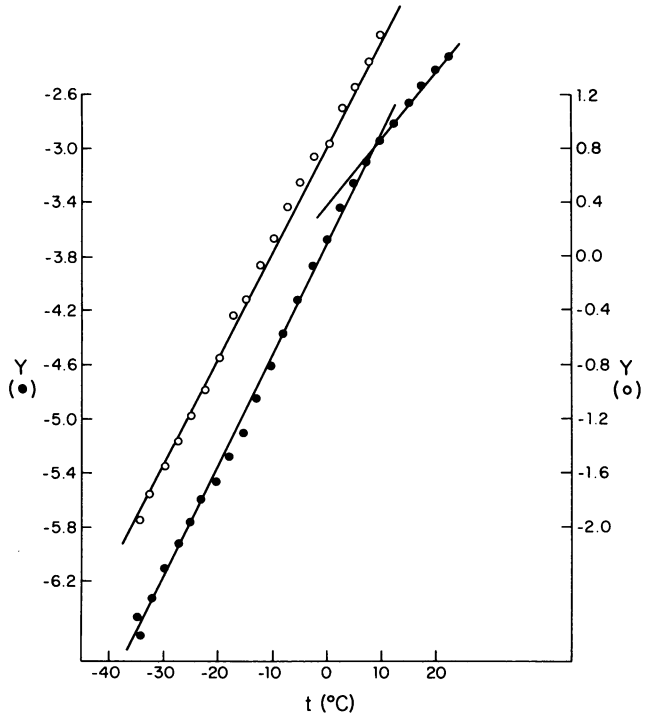

FIGURE 1 The Y versus temperature plot of gramicidin (*solid circles*) and 9-methylanthracene (*open circles*) dissolved in 80% glycerol-ethanol (wt/wt).

To determine the coupling parameters of gramicidin tryptophans to their peptide surroundings, the Y plot in 80% glycerol-ethanol from −35° to 25°C was determined (Fig. 1). The low temperature slope, 8.2 ± 0.01%°C^{-1}, is equal to the value obtained for 9-methyl anthracene (8.2 ± 0.01%°C^{-1}) and by flow viscometry (8.0%°C^{-1}). The critical temperature or the point where **b** changes, is 8 ± 1.3°C. This corresponds to a fluorophore rotational amplitude of 18° when coupling occurs. The high temperature slope is 5.2 ± 0.015%°C^{-1}.

The thermal coefficient of the viscosity of gramicidin in pure ethanol was measured and was found to be approximately 4.0 ± 1.6%°C^{-1}. The high error is due to the low anisotropy values. The **b** value of ethanol taken from tabulated flow viscosity data (CRC Handbook, 56th Ed.) is 2.2%°C^{-1}.

Octylglucoside Micelles

A small amount of gramicidin was dissolved in 55 mM *n*-octylglucoside micelles. The CD spectrum revealed a right-handed species similar to the one shown for the peptide in dipalmititoyl-phosphatidylcholine at low concentrations at 20°C (Sychev and Ivanov, 1982). Attempts to dissolve the micelles in 80% glycerol to observe the fast motions of the tryptophan side chains produced visible aggregation.

The Y plot was determined using the limiting anisotropy value of 0.273 obtained in 80% glycerol-ethanol. The data are presented in Fig. 2 along with *n*-octylglucoside micelles labeled with 12-(9-anthroyloxy) stearic acid. The center of mass of the emission spectrum of anthroyloxy remained constant with temperature while that of the gramicidin showed a 2-nm red shift at 15°C and remained constant thereafter. Below 10°C both gramicidin and the probe start to deviate from a straight line, and these points were not used in the calculation of **b**.

The Y plots in Fig. 2 show that the thermal coefficient of the peptide (**b** = 4.7 ± 0.1%°C^{-1}) is higher than the probe (**b** = 3.4 ± 0.1%°C^{-1}) opposite to the 80% glycerol-ethanol results. This could be due to a contribution of overall micelle rotation to the depolarization, which would tend to decrease **b** because the thermal coefficient of the micelle would be equal to that of the solvent (3.5%°C^{-1}). This

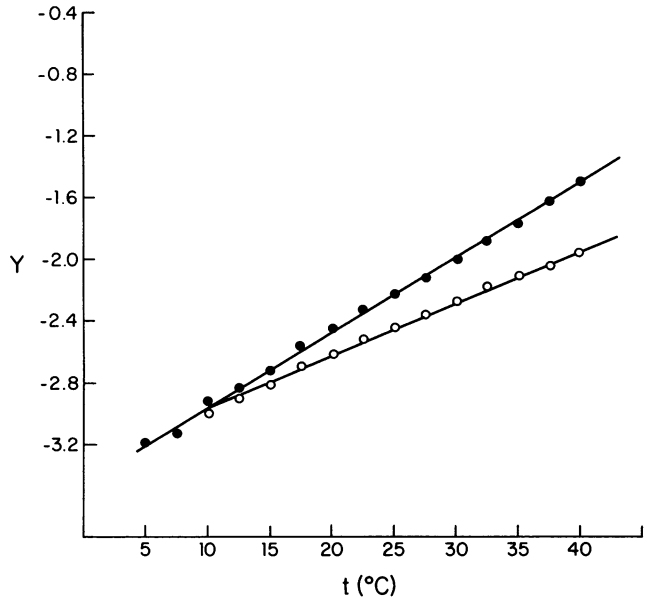

FIGURE 2 The Y versus temperature plot of gramicidin (*solid circles*) and 12-anthroyloxystearic acid (*open circles*) in *n*-octylglucoside micelles.

effect would be more extensive for the longer lived anthroyloxy probe. However, in order for the overall micelle rotation to contribute more than 10% to the anthroyloxy depolarization, the micelles would have to consist of ten molecules or less, which is far lower than those of other uncharged detergents (Helenius and Simons, 1979).

DMPC Bilayers

Gramicidin was incorporated into unilamellar DMPC vesicles using chloroform or ethanol as the cosolvent. Using either cosolvent, the CD spectrum corresponded to the channel form of gramicidin and were identical to those reported by LoGrasso et al. (1988). The fluorescence lifetime and anisotropy were measured from 5° to 40°C and the resulting Y plot is presented in Fig. 3. For comparison, the Y plot of gramicidin-DMPC bilayers labeled with 11-anthroyloxyundecanoic acid is also shown. Below the DMPC phase transition temperature (~25°C) the anisotropy and lifetime of gramicidin did not change ($\mathbf{b} \sim 0°C^{-1}$) even though the viscosity around the anthroyloxy probe changes rapidly with temperature ($\mathbf{b} = 7.8 \pm 0.2\%°C^{-1}$). Above 25°C the anisotropy and lifetime of gramicidin drop yielding a **b** of $8.8 \pm 0.2\%°C^{-1}$ whereas the anthroyloxy probe remains at $7.8 \pm 0.2\%°C^{-1}$. Thus, in micelles and fluid DMPC, gramicidin shows a higher **b** value than the probe even though the CD spectra show that the tertiary structures in these two solvents are different.

It is possible that in the gel phase gramicidin aggregates, causing the tryptophans to be strongly held in place such that they do not respond to temperature changes. Above the DMPC phase transition temperature the aggregate melts thereby causing the viscosity around the fluorophore to change more rapidly than the solvent. Therefore, the experiments were repeated between 28° and 40°C with gramicidin-DMPC bilayers that were kept warm at all times ($T \geq 28°C$). Again, the same **b** value was obtained showing that the high **b** value is not due to the melting of an aggregate formed in the gel phase. Also, if protein does aggregate, we would expect the melting temperature and the higher temperature **b** to depend on the gramicidin concentration. Seven preparations of vesicles having different protein-to-lipid ratios (1:16 to 1:115) were measured and all gave indistinguishable CD spectra and Y plots. Thus, unless the dissociation constant of the aggregate is much smaller, the unusual Y plots are most likely due to intramolecular rather than intermolecular interactions. According to Wallace et al (1978) the CD spectrum of gramicidin does not change with temperature, which implies that the peptide does not undergo gross structural changes in the course of our experiments.

DOPC and eggPC Bilayers

The temperature dependence of gramicidin tryptophan side chain motions was determined for 15 different DOPC samples having protein-to-lipid ratios ranging from 1:50 to 1:123 mol/mol. A hexagonal phase was visible only once, in a sample with a protein-to-lipid ratio of ~1:20 and this solution was disregarded. The CD spectra were identical to those of DMPC showing the same overall protein conformation in the two lipids. None of the samples showed any change in anisotropy ($A = 0.22$) from 5° to 40°C, and the lifetime decreased only slightly. Experiments were repeated with eggPC bilayers to determine whether mem-

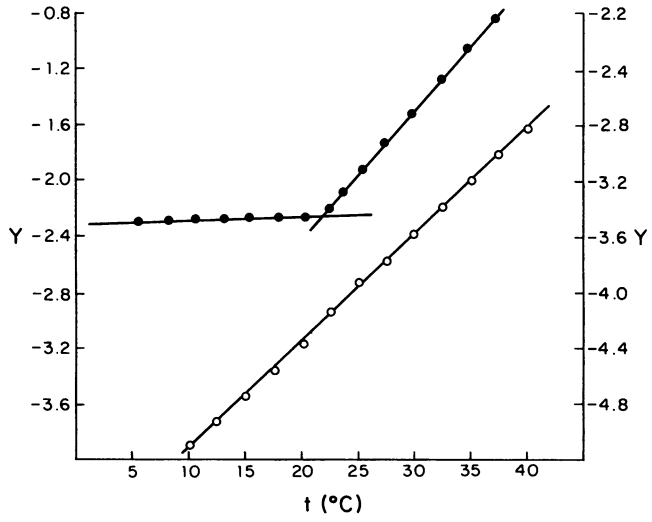

FIGURE 3 The Y versus temperature plot of gramicidin alone (*solid circles*) and gramicidin with 11-anthroyloxyundecanoic acid (*open circles*) incorporated into DMPC bilayers. In the former case the tryptophan fluorescence of the gramicidin was being viewed while in the latter the fluorescence of the anthyroyloxy probe was measured.

branes made from a lipid mixture may alter the anisotropy behavior. However, this was not the case.

Estimation of Ring Stacking

The above data indicate that in some environments (DMPC below 25°C, DOPC, and eggPC) the viscosity around the fluorophore does not change with temperature whereas in others (DMPC above 25°C, micelles), the viscosity around the fluorophore changes more rapidly than that of a probe in the same solvent. One mechanism that could explain this result is that in some situations extensive aromatic ring stacking severely limits the amplitude such that its response to viscosity changes is very low. In certain cases the addition of heat can disrupt stacking, allowing the fluorophore amplitude to increase more rapidly than the external solvent, whereas in others, the ring interactions are much stronger. The amount of stacking can be estimated by comparing the emission intensities exciting at 280 and 305 nm since the probability of homotransfer due to stacking is greatest at the excitation maximum (~280 nm) and lowest on the red edge of the absorption band (Weber, 1960).

The relative quantum yields of gramicidin in octylglucoside micelles and DMPC and DOPC bilayers were measured at two temperatures and compared to a dilute ethanol solution of *N*-acetyl-tryptophanamide (NATA), which does not stack (Table I) and for gramicidin in ethanol. Data were normalized at 35°C and 305-nm excitation. When exciting at 280 nm, extensive photobleaching was observed even at low power levels in accordance with the results of Jones et al (1985). Purging with nitrogen completely eliminated this effect. At 305 nm photobleaching was never observed.

From Table I we see that all samples show the same thermal behavior at 305 nm which is to be expected in a transferless system. Gramicidin in ethanol shows approximately the same intensity values as NATA, whereas in micelles or bilayers more than half of the intensity is lost when exciting at 280 nm. As the temperature increases, the intensities of both NATA and gramicidin in ethanol decrease, and although the 280/305 ratios of GR-DOPC are very different, they too change with temperature in the same proportion. On the other hand, the intensity ratios of gramicidin in micelles and DMPC do not decrease with temperature when exciting at 280 nm. This implies that in these two systems quenching becomes less extensive at higher temperatures.

Based on previous fluorescence studies (Cavatorta et al., 1982), we interpret the quenching of gramicidin tryptophans to be caused by ring stacking and that the change in the amount of quenching with temperature is due to rupture of these interactions. Alternately, changes in the amount of quenching could be caused by changes in the proximity of the tryptophans to lipid or detergent head groups. Although it is unlikely that gramicidin would be affected the same way by both the uncharged, non-

TABLE I
GRAMICIDIN EMISSION INTENSITY RATIOS EXCITING AT TWO WAVELENGTHS

	280/305				
	GR-micelles	GR-DMPC	GR-DOPC	GR-ETOH	NATA
15°C	4.5/1.3	4.0/1.3	2.2/1.3	11.6/1.3	12.5/1.3
35°C	4.8/1.0	4.4/1.0	1.7/1.0	9.0/1.0	9.0/1.0

Intensity data correspond to the integrated corrected emission spectra scanning from 320 to 450 nm. All data are normalized to 305-nm excitation at 35°C. Error on each data point was <0.05.

aromatic sugar groups in the micelle and the phosphatidycholine groups of just DMPC and not DOPC, this possibility was tested by adding *n*-octylglucoside to the gramicidin ethanol solution. No changes in intensity were observed. Therefore, the most likely quenching mechanism is stacking of aromatic rings.

Lifetime Data

Average lifetime measurements $\langle \tau \rangle$ were made using an excitation wavelength of 305 nm for gramicidin and 381 nm for 11-AU. In DMPC vesicles, τ values of both the peptide and probe decreased only slightly (5 and 7%, respectively) below the phase transition temperature from 5° to 25°C but dropped rapidly (27 and 35%, respectively) from 25° to 40°C. In all other systems (micelles, DOPC vesicles, and 80% glycerol-ethanol), the lifetime decreased smoothly with temperature.

At room temperature, the average lifetime of DOPC and eggPC are 2.46 and 2.42 ns, respectively, while DMPC and micelles are 3.46 and 3.38 ns. Thus, even though the overall protein conformation as determined by CD is the same in both DOPC and DMPC, the tryptophan residues experience different environments. This is also apparent from the 25°C anisotropy values (0.22 for DOPC and 0.18 for DMPC).

Preliminary heterogeneity analysis show that in the above solvents gramicidin has at least three lifetime components in agreement with the results of Masotti et al. (1986). Although the fractional contribution of each of these components is similar in all the solvents, the values of all three lifetimes are considerably shorter in eggPC and DOPC than they are in micelles or DMPC. A detailed heterogeneity analysis will be the subject of a future study.

Preliminary time resolved anisotropy data for gramicidin in the different solvents at room temperature indicated a very fast (<800 ps) and a very slow rotational rate. Unfortunately, both these components were out of scale of accurate measurements. It was found that raising the temperature did not significantly change these rates in DMPC. Also, because of the rapid depolarization, we were unable to obtain the limiting anisotropy of these solutions by extrapolation to zero time.

DISCUSSION

Initially, the temperature dependence of gramicidin tryptophan rotations were observed in the isotropic, viscous solvent, 80% glycerol-ethanol since 80% glycerol-water was previously used to investigate fluorophore motions in globular peptides and proteins. In ethanol, the gramicidin dimer is an antiparallel double helix (Veatch et al., 1974) and we expect the protein to have a similar structure in 80% glycerol-ethanol. The tryptophan motions of gramicidin behave like those of aqueous peptides in that the Y plot displays two **b** values: One at lower temperatures equal to the external solvent and another at higher temperatures due to restriction to fluorophore rotation caused by the peptide environment. The values of the critical temperature and the high temperature **b** value are higher than most globular peptides and proteins but close to the ones obtained for tropomyosin, a hexamer consisting of two intertwined alpha helices whose tyrosines protrude out into the solvent (Phillips et al., 1979). The critical temperature relates to the fluorophore amplitude that must be reached in order for motion to become limited by the surrounding peptide. Analogous to tropomyosin we interpret the high critical temperature as being the result of the large degree of solvent exposure of the tryptophans due to the helical conformation of the peptide. This configuration also results in changes in fluorophore amplitude with temperature that follow the solvent to a greater extent yielding a high **b** value. This interpretation correlates well with the recent crystal structures of Wallace and Ravikumar (1988) and Langs (1988).

The temperature dependence of gramicidin tryptophan rotations in micelles and bilayers differ markedly from the isotropic solvent. One factor that may account for the differences in rotational behavior is the assumption that the limiting anisotropy, A_0, remains the same in going from 80% glycerol-ethanol to micelles and to bilayers even though the CD spectra show different conformations in all these solvents. Differences in A_0 would effect the values of both Y and **b** in our calculations. Attempts to measure A_0 in micelles and bilayers by time-resolved techniques fail due to very fast motions during the first few picoseconds. Thus, in order to determine how changing the limiting anisotropy affects **b**, we calculated the Y plot for micelles using different limiting anisotropies. Based on surveys of the limiting anisotropies of globular proteins (Scarlata, 1984), A_0 was varied by 20%. Increasing A_0 by 20% from 0.273 where ($\mathbf{b} = 4.7\%°C^{-1}$) to 0.333 gave a linear Y plot with a higher **b** value ($5.8\%°C^{-1}$). Lowering A_0 by 20% to 0.22 also lowers **b** to $4.2\%°C^{-1}$. Since all these values are greater than that of the probe ($3.4\%°C^{-1}$), unless the limiting anisotropy of the anthroyloxy probe increases in going from 80% glycerol-ethanol to micelles and its **b** value increases, which is unlikely, the gramicidin **b** values are higher than the anthroyloxy fatty acid.

Another factor that could explain the different tryptophan temperature behavior in 80% glycerol, micelles, and membranes is a change in the rotational rate of the fluorophore since this would alter the total amplitude experienced by the fluorophore during its lifetime. For example, if the rotational rate is slow enough at lower temperatures such that the fluorophore does not fill up all the space allowed by the solvent, but the rate rapidly increases with heat, then the change in amplitude with temperature is not only due to the relief of restriction from the solvent but also to the increased rate. Preliminary time-resolved measurements indicate a very fast rotational motion and a very slow one, and in DMPC, these rates do not change greatly with temperature. The fast motion may reflect the vibrations of the tryptophan residues and the slow one probably corresponds to larger peptide and/or bilayer motions. Simulations of Y plots show that the fluorophore rotational rate must be very slow in order to significantly change the value of **b** (Scarlata, unpublished results). Thus, we conclude that gramicidin tryptophans rotate at a sufficient rate to fill the space permitted by their environment in accordance with previous findings (Scarlata et al., 1984).

It therefore appears that different solvents can modulate different gramicidin conformations which in turn modulate different protein–tryptophan interactions. In eggPC, DOPC, and gel-phase DMPC vesicles, tryptophan side chains do not sense fluidity changes that are occurring in the membrane surroundings, whereas in fluid DMPC and micelles the tryptophan amplitudes decrease rapidly with temperature. We first focus on the DOPC results. According to the mechanism proposed by Killian and de Kruijff (1988), gramicidin is able to induce a bilayer to hexagonal phase transition in longer unsaturated lipids through protein association promoted by intramolecular aromatic stacking. Under the experimental conditions used here (hydrated bilayers, protein/lipid molar ratios averaging 1:50), the structure of our gramicidin-DOPC of eggPC mixtures should have undergone the separation step but not the transition to the hexagonal phase. This conclusion is supported by the CD spectrum that showed the gramicidin to be in the channel conformation, and the hexagonal phase was never visibly observed.

The fluorescence parameters of gramicidin in DOPC and eggPC all indicate a temperature-stable protein aggregate: extensive energy transfer due to tryptophan stacking, a very short lifetime, and a high anisotropy that remains constant with temperature. Spisni et al (1983) proposed a hexamer structure for the gramicidin aggregates that form in lysophosphatidylcholine stabilized by intermolecular tryptophan contacts on each of the three gramicidin apices. The results show that the tryptophan rings are trapped into a small vibrational area by extensive aromatic ring stacking. Based on the work of Spisni et al. (1983), we conclude that the complex is held together by intermolecular tryptophan contacts.

The anisotropy behavior of gramicidin in gel-phase

DMPC also shows that the gramicidin tryptophans are locked into fast, small amplitude motions that do not respond to temperature changes due to ring stacking. Comparing the gel DMPC anisotropy, lifetime, and energy transfer values to those obtained for DOPC, eggPC, and ethanol, it appears that stacking exists (presumably between trps 9 and 15) in DMPC but it is not as extensive as in DOPC or eggPC. The behavior of gramicidin in gel-phase DMPC cannot be attributed to extensive ring interactions as was the case for longer chain lipids unless trps 9 and 15 constitute most of the observed fluorescence intensity. Mechanisms that may be responsible for holding the tryptophans in place are either small scale intermolecular tryptophan interactions or a very high local viscosity coupled with a short rotational rate or a combination of both. We shall now try to differentiate between these possibilities.

Adding heat to the system produces a rapid drop in the DMPC viscosity as the fluid phase is reached (e.g., see Lentz et al., 1976). This decrease in lipid ordering is readily seen in the rapid drop in lifetimes of both the probe and peptide after the phase transition presumably due to the large increase in thermal de-excitation pathways. We note that the Y analysis takes into account changes that occur in fluorescence lifetime and pertains only to changes in rotational freedom.

The energy transfer data of gramicidin in DMPC coupled with the anistropy and lifetime values show that in the fluid phase, ring interactions are disrupted thereby causing tryptophan amplitude to increase more rapidly with temperature than if the tryptophan residues were isolated. Although this result may indicate that the ring interactions are intermolecular, the fluorescence data of gramicidin in micelles give the same results. Thus, unless the *n*-octylglucoside detergent was only able to solubilize the protein at higher temperatures, the gel-phase DMPC results are explained by high viscosity which greatly restricts rotational motions and, in the fluid phase, by a breaking of trp 9 and 15 interactions as the temperature is raised. The increased mobility of the tryptophans at higher temperatures may influence transport through the channel as observed in recent ^{23}Na-NMR studies (Buster et al., 1988). The energy transfer results also indicate a significant amount of stacking is still present at 35°C.

When comparing our DMPC results to those obtained in other environments, it appears that since the micelles are more fluid, the fluorophore amplitudes are less locked-in and thus more responsive to viscosity changes. In DOPC and eggPC, a larger number of fluorophores can participate in stacking and thus the enthalpy needed to break these interactions is greatly increased. We can compare the DMPC results to recent deuterium NMR studies (Macdonald and Seelig, 1988) which have shown that in gel phase, gramicidin is essentially immobile but above the phase transition temperature, large scale rotational fluctuations of the protein occur. Correlating these results for overall protein rotation to ours for local tryptophan motions, we interpret the phase transition temperature as the point where the lipid is fluid enough to allow for greater rotational amplitude of the aromatic rings. We note that in the channel conformation, the tryptophan side chains are close to the membrane surface and are not expected to experience large differences in membrane viscosity (Urry, 1971). The existence of a temperature region where fluorophore rotations are unresponsive to viscosity decreases has also been observed for porphyrin motions in the heme pocket of myoglobin (Royer and Alpert, 1987).

Since gramicidin has a different tertiary structure in 80% glycerol-ethanol than in DMPC or DOPC (Urry, 1971; Wallace and Ravidumar, 1988; Langs, 1988), a direct comparison of the rotational motion in a viscous, ordered lipid state to a viscous, isotropic solvent is not possible. We can, however, compare the rotational behavior of gramicidin tryptophans in vesicles to that of the tyrosine of vasopressin in 80% glycerol-water since the fluorophore motions in the latter case are also dictated by a strong ring stacking interaction with a neighboring phenylalanine (Scarlata et al., 1984). Whereas in the highly viscous gel-phase DMPC the motions of the ring-stacked complex do not respond to viscosity changes, the fluorophore of vasopressin is responsive to the changes occurring in the solvent even though the change in viscosity with temperature is approximately the same in both systems as seen by anthroyloxy probes. One explanation could be that the ring interactions in the gramicidin channel are much stronger than those of vasopressin due to either the intrinsic properties of the fluorophores, stabilization by the lipid of a peptide conformer whose stacking interactions are strong, or stabilization of the stacked complex due to the protrusion of the tryptophans into the membrane. Alternately, the ordering of lipid in gel phase around the peptide could be extensive enough such that a lipid–peptide aggregate forms and only after the phase transition temperature does the complex melt and allow the tryptophan motions to sense the viscosity changes occurring in the membrane. Since not all the tryptophans participate in ring stacking, the latter mechanism is most likely.

The interpretation of the data obtained in this study implies that $\mathbf{b}$ is related to the thermal expansion coefficient (β) of condensed solvents and we note that both $\mathbf{b}$ and β have the same units (1/temperature). Therefore, assuming that the expansion of fluorophore amplitude is similar to isotropic liquids and solids we can write

$$d(\mathrm{AMP}) = \mathbf{b} \cdot \mathrm{AMP}_o \cdot dT,$$

where AMP_o is the amount of fluorophore rotational amplitude at the initial temperature point. For a freely diffusing molecule, the rotational amplitude will vary from 0° to 90° for no rotation and complete rotation, respectively. The anisotropy is determined from the amplitude by

$$2/(3\cos^2 \mathrm{AMP} - 1) = A_o/A.$$

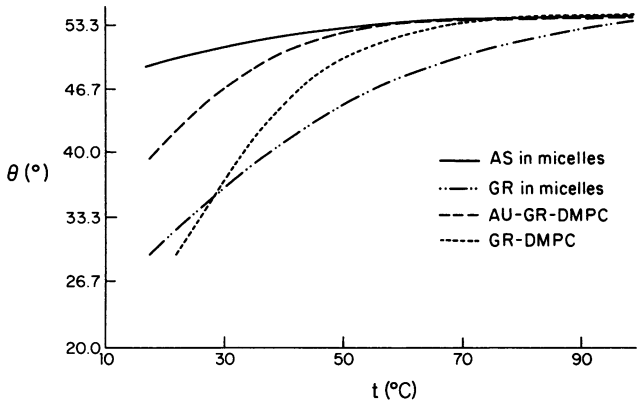

FIGURE 4 Computer-generated plot of the change in rotational amplitude (θ) with temperature calculated from the input parameters of 12-anthroyloxystearic acid (AS) in *n*-octylglucoside micelles, gramicidin (GR) in *n*-octylglucoside micelles, 11-anthroyloxyundecanoic acid (AU) and GR in DMPC bilayers, and GR-DMPC bilayers.

Using the above equations we can generate Y plots identical to those obtained experimentally. Also, we can test the expansion by varying the input parameters of AMP_o, **b**, and the temperature range and we find that this analysis is self-consistent within 10%. The above expansion can be verified by comparing the values of **b** to thermal expansion values of various solvents. Extracting these data from the CRC Handbook (50th Ed.) a linear relation appears to exist for several organic solvents. The notable exception is water, which has a negative thermal expansion. Thus we conclude that, to a first approximation, **b** can be thought of as the thermal expansion coefficient of the fluorophore environment.

Changes in fluorophore amplitude with temperature generated by the above equations are shown in Fig. 4 using the experimental **b** and AMP_o of gramicidin in micelles and DMPC along with the anthroyloxy probes in micelles and DMPC for comparison. First note that the amplitude increases until the magic angle (55°) where the anisotropy becomes zero. The difference in the rate of change in amplitude between the probe and protein should be related to the amount of ring stacking disrupted with temperature.

From this study we conclude that membrane viscosity does serve to regulate the motions and peptide interactions of the integral membrane protein gramicidin, but not in a straightforward manner. Work is under way to determine whether other integral membrane proteins show similar behavior.

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