Kinetics of channel formation of gramicidins A and B in phospholipid vesicle membranes

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ABSTRACT The thermal incorporation and channel formation of gramicidins A and B into phosphatidylcholine/phosphatidylglycerol large unilamellar vesicle membranes was studied using ^{23}Na NMR. ΔH and ΔS of activation for channel formation for gramicidin A are 11.8 kcal/mol and −11 e.u., respectively. For gramicidin B, ΔH and ΔS of activation are 14.6 kcal/mol and −4 e.u., respectively. Possible reasons for the differences in ΔH and ΔS of activation between the two analogues are discussed.

INTRODUCTION

Gramicidin is a 15–amino acid linear polypeptide which forms monovalent cation-conducting channels in lipid membranes (Meyers and Haydon, 1972; Anderson, 1983). The amino acid sequence of gramicidin *A* is: formyl-L-Val^1-Gly^2-L-Ala^3-D-Leu^4-L-Ala^5-D-Val^6-L-Val^7-D-Val^8-L-Trp^9-D-Leu^{10}-L-Trp^{11}-D-Leu^{12}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15}-ethanolamine. The channel consists of two monomeric beta helices joined by hydrogen bonds at their NH_2 terminal ends (Urry, 1971; Szabo and Urry, 1979; Bamberg et al., 1977; Weinstein et al., 1979, 1980). The dimer's length is 26 Å and forms a 4-Å pore and is shown in Fig. 1. Gramicidin B differs from gramicidin A by a phenylalanine at position 11 in place of a tryptophan (Sarge and Witkop, 1965).

The transport of sodium ions by gramicidin across bilayer membranes of large unilamellar vesicles (LUV) under conditions of ionic equilibrium can be studied using ^{23}Na NMR (Buster et al., 1988). This is possible because the transport of sodium ions across the vesicle wall by active gramicidin dimers results in a change in the linewidth of the NMR signal. The linewidth of the NMR signal is proportional to the square of the gramicidin monomer concentration (Buster et al., 1988), since the channel is a dimer.

$$1/\tau = \pi\Delta\nu_{1/2} = k[\text{gramicidin}]^2, \qquad (1)$$

where τ is the mean lifetime of the Na^+ inside of the LUV, $\Delta\nu_{1/2}$ is the linewidth at half-height of the inside ^{23}Na NMR signal, and k is the rate constant for the transport process. The spectra in Fig. 2 show the effect of varying amounts of gramicidin D (a mixture of gramicidins 80% A, 5% B, and 15% C) on the ^{23}Na NMR signals in the vesicle system when a chemical shift reagent is added to the solution outside the vesicles (Buster et al., 1988).

We wish to report the results of a ^{23}Na NMR investigation of the thermal incorporation and channel formation of gramicidin analogues A and B into large unilamellar vesicles under ionic equilibrium conditions using ^{23}Na NMR. The incorporation of conducting gramicidin channels into the vesicle membrane under ionic equilibrium conditions is not spontaneous but requires a thermal incubation period. In a nonequilibrium system the incorporation of gramicidin is spontaneous, driven by transmembrane potential effects (Buster et al., 1988). Line broadening occurs as the number of conducting dimers in the lipid membrane increases with time of thermal incubation. The rate constant for channel formation of gramicidin in the lipid membrane can be determined at different incubation temperatures and the thermodynamic parameters calculated for the process.

The NMR technique is potentially useful for exploring the effect of single amino acid substitution in gramicidin on the rate of formation of a viable channel in a lipid membrane. The effect of different lipids or membrane constituents (i.e., cholesterol) on the process can also be studied using this technique. The only requirement for a molecule to be studied in this way is that the molecule must conduct ions at a rate sufficiently fast to cause a change in the linewidth of the NMR signal of the conducting ion.

The incorporation of gramicidin into lysolecithin micelles has been investigated previously by ^{13}C NMR and was found to be irreversible (Spisni et al., 1979). This technique, however, does not isolate one particular gramicidin structure in the membrane. With the ^{23}Na NMR technique only active conducting channels are measured.

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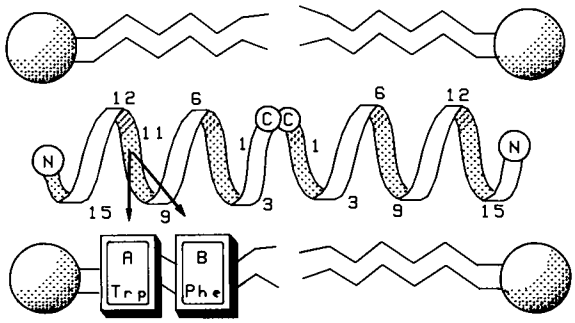

FIGURE 1 Gramicidin dimer in a lipid bilayer membrane.

The incorporation of gramicidin into lysophosphatidylcholine micelles was also studied using fluorescence measurements (Cavatorta et al., 1982). With the NMR method the peptide does not have to have a fluorescence or absorbance spectrum. The incorporation rate of gramicidin into lysolecithin micelles has also been shown to be dependent upon the initial concentration of gramicidin (Masotti et al., 1983), but the specific dependence was not determined.

METHODS

Phase transition

Gramicidin requires a thermal incubation period above the lipid phase transition temperature in order to incorporate into the vesicle membrane. Not having calorimetric data for the phase transition temperature of the phosphatidylcholine (PC)/phosphatidylglycerol (PG) vesicles, ^{31}P NMR of the lipid head groups was used to estimate the value. It has been shown that ^{31}P NMR linewidths are sensitive to phase transitions of phospholipid membranes (McLaughlin et al., 1975). From Fig. 3, which contains the linewidth as a function of temperature data, a value of 42°C was obtained for the phase transition temperature. Although values measured in this way may vary from the true transition temperature by a few degrees (McLaughlin et al., 1975), the value obtained for the phase transition temperature is sufficiently accurate to be used for the incorporation experiments.

LUV samples for ^{31}P NMR spectra were prepared as before (Buster et al., 1988) except 10 mM tris(hydroxymethyl)aminomethane was used instead of K_2HPO_4/H_3PO_4 to buffer the system to a pH 7.8. Also, the vesicle solutions were not diluted with shift reagent. This was done to eliminate other ^{31}P signals in the NMR spectrum. ^{31}P Spectra were recorded at 36.23 MHz. 1,000 FIDs were accumulated with a delay time of 1 s.

Incorporation

The LUV samples (2.5–3.5 ml), prepared as before and filtered to produce a homogeneous solution of vesicles (Buster et al., 1988), were thermally equilibrated at the desired temperature (45–70°C) in a constant temperature bath (±0.1°C). A known concentration of gramicidin in trifluoroethanol (TFE) was added to each sample with a microliter syringe to give a lipid/gramicidin ratio of 11:1. After mixing with a vortex mixer the sample was placed immediately back into the temperature bath. Aliquots of 0.5 ml were then taken as a function of time and placed into a 10-mm NMR tube which was then placed in an ice bath for 15 min to quench the incorporation process. The NMR samples were diluted 1:1 with an aqueous shift reagent of $Dy(P_3O_{10})_2^{-7}$ such that the final concentration of the shift reagent present on the outside of the vesicles was 5 mM and there was an ionic balance between the inside and outside aqueous pools. The solutions were stored overnight at 4°C and then allowed to equilibrate to the NMR probe temperature before data acquisition. A LUV sample without gramicidin was treated similarly and used as a blank. ^{23}Na spectra were recorded at 23.65 MHz on a spectrometer (model FX90Q; Jeol USA, Analytical Instruments Div., Cranford, NJ) using a 10-mm multinuclear probe. Typically, 1,000 FIDs were accumulated per data set at a probe temperature of 27°C, where incorporation is negligibly slow. The linewidth at half height was determined for the NMR signal of the Na ions on the inside of the vesicles. The change in linewidth for each

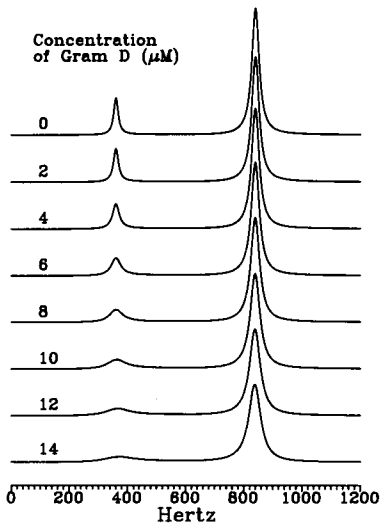

FIGURE 2 Gramicidin *D* concentration dependence upon the inside and outside 23.65 MHz ^{23}Na NMR signal.

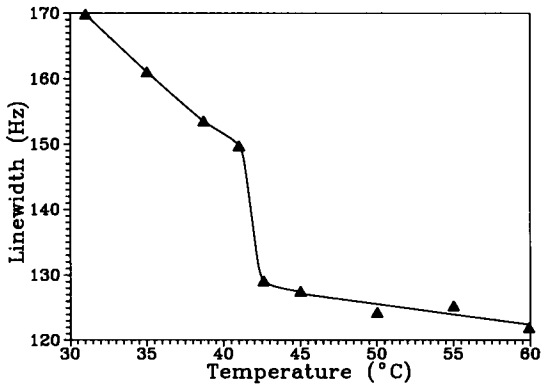

FIGURE 3 36.23 MHz ^{31}P NMR linewidth of PC/PG vesicles as a function of temperature.

sample was calculated by subtracting the linewidth of the blank from the linewidth of the sample.

RESULTS

Fig. 4 shows the time course of the increase in the change in linewidth of the inside ^{23}Na NMR signal while heating at various temperatures after the addition of gramicidin A. This clearly shows that the rate of incorporation and channel formation of gramicidin A in the lipid membrane is temperature dependent. Similar results were also obtained for gramicidin B.

The question arises as to whether the vesicles are stable when heated above the phase transition or if they are stable over long periods of time. To answer these questions, vesicles were prepared as before and filtered. Half of the LUV solution was incubated for 3 h at 65°C and the other half was left at room temperature. Then both samples were again filtered and diluted with shift reagent and the NMR spectra were taken. By determining the areas contained by the NMR signals of both the inside and outside pools of Na^+ the percent encapsulation of Na^+ can be calculated (Buster et al., 1988). It was assumed that if the vesicles were unstable to heating they would either form larger vesicles by fusion of the vesicles or decompose into sheet type structures. If either or both cases occurred, after filtering, a reduction in encapsulated Na^+ should be seen. However, the percent encapsulation was the same for both the incubated and nonincubated samples. When the samples were left at room temperature for a period of 5 d no change was seen in the percent encapsulation.

To determine if the incorporation process is reversible, an incorporation experiment was altered slightly. A sample was heated at 70°C for 45 min and aliquots were analyzed as before. Then the sample was placed in a 45°C bath and again aliquots were analyzed as a function of time. Two different samples of the same solution were also heated, one at 45°C and the other at 70°C, for comparison. The results of this experiment are shown in Fig. 5. If the process is reversible the sample heated for 45 min at 70°C and then heated for several hours at 45°C would be expected to show a decrease in the change in linewidth upon heating at 45°C and approach the line for the sample heated only at 45°C. This is not the case, as shown in Fig. 5. The linewidth does not approach that of the one at 45°C but continues to increase, demonstrating the nonreversible nature of the incorporation process. Thus, once the monomers are in the membrane they remain available to form conducting dimers. The irreversible nature of gramicidin incorporation into micelles was previously demonstrated (Spisni et al., 1979), which supports our results. This is to be expected due to the insolubility of gramicidin in water.

There is evidence for the formation of gramicidin aggregates on the surface of lysophosphatidylcholine micelles (Cavatorta et al., 1982) and phospholipid vesicles (Kemp and Wenner, 1976) before heating. The formation of aggregates is primarily the result of the low solubility of gramicidin in water due to the hydrophobic amino acids making up the sequence of gramicidin (Haydon and Hladky, 1972). It was proposed that incorporation occurs from the aggregates at the lipid/water interface. The aggregates are probably β-helical dimers associated with the lipid surface (Kemp and Wenner, 1976). The dimers then dissociate into monomers. Once incorporated, the formation of an active channel of gramicidin in a membrane is a reversible second-order process (Bamberg and Lauger, 1973; Buster et al., 1988). In TFE gramicidin forms β-helical type dimers (Urry et al., 1975)

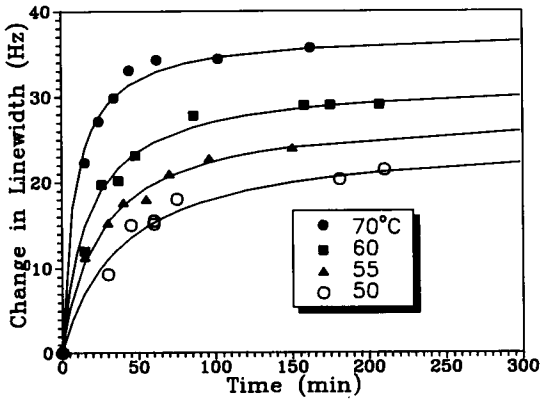

FIGURE 4 Time course for the change in linewidth of the inside ^{23}Na NMR signal for various incubation temperatures for gramicidin *A*.

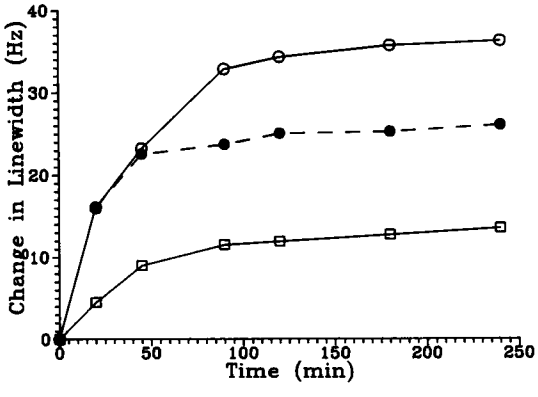

FIGURE 5 Result of experiment to demonstrate the irreversible nature of the incorporation process of gramicidin *A*. (○) 70°C, (□) 45°C, and (●) sample heated for 45 min at 70°C, then for the remaining time at 45°C.

and when added to the vesicle solution it is possible that a similar structure is maintained at the water/solvent/membrane interface.

We propose a simple mechanism for the thermal incorporation and channel formation of gramicidin in vesicle membranes above the lipid phase transition. Gramicidin dimers, in TFE, added to the solution of vesicles associate with the surface of the vesicles and are irreversibly driven into the bilayer membrane by thermal incubation. While being driven into the membrane the dimers dissociate with the monomers being distributed predominantly in the outer half of the bilayer membrane. The monomers then "flip-flop" into the other side of the membrane, where they dimerize to form active conducting channels. This would account for the halt in the further forming of channels observed once the vesicles are brought below the phase transition temperature. It was interpreted from single channel conductance experiments that the monomer is the dominant form for the association of gramicidin into the membrane (Maddock et al., 1989); however, it is possible that a mechanism such as we propose might account for their observation. The scheme is represented by the following expressions:

$$\mathrm{Gr}^{\mathrm{H}}_{2(\mathrm{as})} \xrightarrow{k_1} \mathrm{Gr}_{(\mathrm{i})}, \tag{2}$$

$$\mathrm{Gr}_{(\mathrm{i})} \underset{k_3}{\overset{k_2}{\rightleftharpoons}} \mathrm{Gr}^{\beta}_{2(\mathrm{i})}. \tag{3}$$

$\mathrm{Gr}^{\mathrm{H}}_{2(\mathrm{as})}$ represents the aggregates of gramicidin helical dimers associated with the surface of the membrane. $\mathrm{Gr}_{(\mathrm{i})}$ represents the gramicidin monomers present in the outer and inner halves of the bilayer membrane, respectively. The conducting dimer is represented by $\mathrm{Gr}^{\beta}_{2(\mathrm{i})}$. The rate constant for the incorporation of gramicidin into the membrane is k_1. The rate constant for dimerization is k_2, and the dissociation rate constant is k_3.

Single channel studies show that the equilibrium constant for channel formation is quite large ($K = 1.5 \times 10^{14}$ cm^2/mol) (Bamberg and Lauger, 1973). Further studies show that gramicidins A and B have similar channel lifetimes (Bamberg et al., 1976); therefore, we assume that their equilibrium constants are of the same order of magnitude. We assume that the back reaction (dissociation), because of the large equilibrium constant, does not occur to an appreciable extent during the course of the experiment. This results in the following scheme:

$$\mathrm{Gr}^{\mathrm{H}}_{2(\mathrm{as})} \xrightarrow{k_1} 2\mathrm{Gr}_{(\mathrm{i})} \xrightarrow{k_2} \mathrm{Gr}^{\beta}_{2(\mathrm{i})}. \tag{4}$$

Setting $[\mathrm{Gr}^{\mathrm{H}}_{2(\mathrm{as})}] = a_o - \chi_t$, $[2\mathrm{Gr}_{(\mathrm{i})}] = \chi_t - \gamma_t$, and $[\mathrm{Gr}^{\beta}_{2(\mathrm{i})}] = \gamma_t$, the following equations can be written:

$$d_{\chi_t}/dt = k_1(a_o/2 - \chi_t) \tag{5}$$

$$d_{\gamma_t}/dt = k_2/2(\chi_t - \gamma_t/2)^2. \tag{6}$$

From these the following expression for the production of γ_t (conducting dimers) is obtained, with $b = a_o k_2/k_1$ and $a_o = 3 \times 10^{-6}$ mol/dm^3:

$$\gamma_t = a_o(1 - e^{-k_1 t})[bk_1 t - b + be^{-k_1 t}/ (1 + bk_1 t - b + be^{-k_1 t})]. \tag{7}$$

For a more detailed derivation see Bamford and Tipper (1969). To get the relationship between the change in linewidth and the dimer concentration we use the following argument. The total concentration, N, of gramicidin in the membrane is related to the concentration of monomers, N_1, and dimers, N_2, by:

$$N = N_1 + 2N_2. \tag{8}$$

And the fraction, y, of dimers, is:

$$y \equiv N_2/N. \tag{9}$$

Using Eq. 8 it can be shown that the maximum value of y (i.e., $N_1 = 0$) is 0.5, which represents complete dimerization of the gramicidin in the membrane. y can be calculated (Bamberg and Lauger, 1973) using:

$$y = 1/8NK(1 + 4NK - \sqrt{1 + 8NK}). \tag{10}$$

Thus, when $K = 1.5 \times 10^{14}$ cm^2/mol, (Bamberg and Lauger, 1973) and at our lipid/gramicidin ratios $N \simeq \times 10^{-12}$ mol/cm^2, $y \simeq 0.5$. Though the value of $K = 1.5 \times 10^{14}$ cm^2/mol may not be absolutely valid for our system, it would seem that at our high gramicidin/lipid ratio and with the membrane below the phase transition temperature, $y \simeq 0.5$ would be a valid assumption. Therefore, at $t = \infty$, $\pi\Delta\upsilon_{1/2(\infty)} = k_t[\mathrm{Gr}^{\beta}_2]_{(\infty)}$, where $[\mathrm{Gr}^{\beta}_2]_{(\infty)} \simeq a_o$ and k_t is the rate constant for the transport of Na^+ through the gramicidin channel. After calculating k_t from the maximum linewidth value, then χ_t can be calculated from Eq. 11.

$$\gamma_t = \pi\Delta\upsilon_{1/2(\mathrm{i})}/k_t. \tag{11}$$

By using Eq. 7 and altering k_1 and k_2, the values for the rate constants can be found by fitting calculated changes in linewidth with the experimentally obtained values (Table 1). Attempts to fit the data to any first order scheme were unsuccessful. An induction period is seen when the calculated dimer concentration is plotted against time when k_1 is sufficiently small (Fig. 6), which is not observed in the experimental data. Thus, a best fit of all the data is obtained for values of $k_1 > 0.1$ s^{-1}. Due to the exponential term in Eq. 7 this value could not be

TABLE 1 Sample result of a rate constant calculation for gramicidin B

Time	Experimental linewidth	Calculated linewidth	Deviation
s	*Hz*	*Hz*	*Hz*
0	0.0	0.0	0.0
300	4.8	4.8	0.0
900	10.0	9.3	0.7
1,800	11.7	12.0	0.3
3,600	13.7	14.1	0.4
5,400	15.2	15.0	0.2
7,200	16.1	15.4	0.7
		Average deviation	0.33

k_2 = 450 dm^3 mol^{-1} s^{-1}. a_o = 3 × 10^{-6} mol dm^{-3}. $\Delta\nu_{1/2(\infty)}$ = 17 Hz. The average deviations between the experimental linewidths and the calculated linewidths for both analogues ranged from 0.29 to 0.89 Hz.

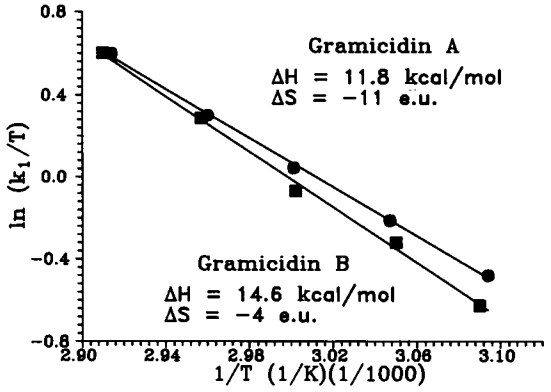

FIGURE 7 Plot of $-ln\,(k_2/T)$ vs. $1/T$ for the channel-forming process for gramicidins *A* and *B* in the vesicle membrane.

accurately determined for higher values. It is tempting to try to relate our rate for channel formation with those previously published (Bamberg and Lauger, 1973). However, this is not possible for several reasons: (*a*) our rate constants are determined above the phase transition where gramicidin is free to move across the bilayer; (*b*) the vesicle membrane is not planar but is curved, which would affect the mobility of the monomers and hence association; (*c*) the phospholipids are different; and (*d*) higher gramicidin/lipid ratios are used in our experiment.

From a plot of $-\ln(k_2/T)$ vs. $1/T$ (Fig. 7), the ΔH and ΔS for channel formation of gramicidin for each analogue can be determined. ΔH and ΔS of activation for channel formation by gramicidin A are 11.8 ± 0.5 kcal/mol and −11 ± 2 e.u. (at 25°C), respectively. For gramicidin B, ΔH and ΔS of activation are 14.6 ± 0.4 kcal/mol and −4 ± 2 e.u. (at 25°C), respectively.

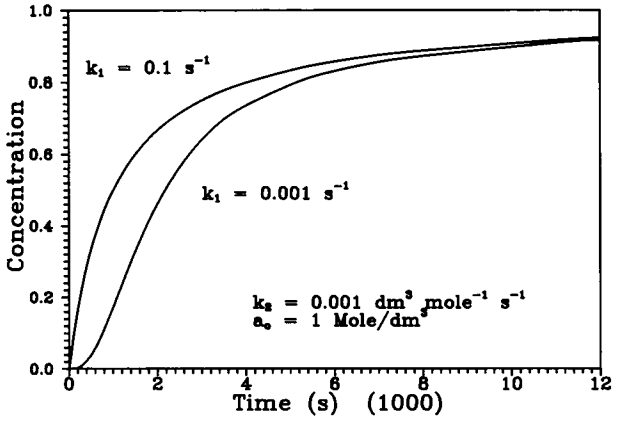

FIGURE 6 The effect of k_1 upon the conducting dimer concentration as a function of time, with k_2 constant. Notice the induction period when k_1 = 0.001 s^{-1}.

DISCUSSION

The differences in ΔH and ΔS of activation found between gramicidins A and B show that single amino acid substitutions can have a significant effect on the rate of channel formation of gramicidin. There are two possible factors which can influence the incorporation and channel formation of gramicidin in the lipid membrane; the hydrophobicity of the amino acid side chains and interactions of the side chains with the lipid molecules.

Tryptophan–tryptophan and tryptophan–lipid interactions play an important role in gramicidin–lipid organization and gramicidin's ability to self associate. The tryptophans are known to be essential for gramicidin's ability to modulate lipid structure (Killian et al., 1985). Fluorescence studies show that tryptophan residues play an important part in the incorporation of gramicidin into lysophosphatidylcholine micelles and the ability of gramicidin to induce the formation of bilayers with lysoPC (Cavatorta et al., 1982; Masotti et al., 1986). Gramicidin B exhibits a reduction in the capability of the peptide to modulate lipid structure compared with gramicidin A (Killian et al., 1985). Such results demonstrate the importance of these side chain interactions in determining lipid–gramicidin structure. The lipid–side chain interactions could play an important role in the incorporation process. When the temperature is raised above the phase transition temperature (~42°C) so that the membrane is in a "liquid" phase, gramicidin is able to incorporate into the membrane. The phase transition of the lipid membrane involves the disruption of intermolecular bonds among the polar head groups of the lipids (Tanford,

1980). It is interesting to note that many biological membranes are in this "liquid" state at ambient temperatures (Lee, 1977; Cullis and DeKruijff, 1979). To incorporate into the membrane gramicidin must disrupt the lipid–lipid bonds further. Through lipid–side chain interactions gramicidin is able to compensate for the disruption of the lipid–lipid bonds caused by incorporating the polypeptide in the membrane and thus form channels.

Another factor in the incorporation and channel formation of gramicidin into a lipid membrane from an aqueous environment is the hydrophobicity of the amino acid side chains. Tryptophan is a more hydrophobic moiety than is phenylalanine, as measured by the change in free energy of transfer from water to an organic solvent (tryptophan, −3.4 kcal/mol at 25°C; phenylalanine, −2.5 kcal/mol at 25°C) (Nozaki and Tanford, 1971). The difference in hydrophobicity of the substituted side chains would suggest that gramicidin A might incorporate and form channels more readily than gramicidin B. This was found to be true by our ^{23}Na NMR studies. However, care must be taken when applying the hydrophobicity scale to a water/lipid system. There are likely to be specific interactions between hydroxyl and other polar groups of the side chains with the polar head group of the lipid molecule, thus affecting the free energies of transfer (Nozaki and Tanford, 1971).

The negative change in entropy for each analogue involves three factors: (*a*) gramicidin going from an aggregate structure to a more ordered dimer form; (*b*) constraint of the normal motion of the polypeptide and lipid molecules by lipid–peptide interactions; and (*c*) constraint of the motions of the side chains. It would seem that all three factors play a part in determining the difference in entropy between gramicidins A and B. Side chain substitutions could affect aggregate or dimer structure, thus resulting in a different change in entropy. The translational motion of the peptide is constrained to two dimensions in the plane of the bilayer. Side chain/side chain and side chain/lipid interactions could also affect the mobility of the polypeptide and the motions of the side chains. It has been shown that the rotational motion of gramicidin in lipid membranes is slower than in methanol and that tryptophan motions are restricted. The rotational correlation time of gramicidin increases from 10^{-9} s in methanol to 10^{-7}–10^{-6} s in bilayer membranes (Macdonald and Seelig, 1988). Gramicidin A has a ΔS of activation of −11 e.u. and gramicidin B, with one less tryptophan, has a ΔS of activation of −4 e.u. Our results suggest that tryptophan/lipid or tryptophan/tryptophan interactions affect the normal motions of the side chains and the peptide in the lipid membrane.

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