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CHARACTERIZATION OF MICELLAR-PACKAGED GRAMICIDIN A CHANNELS

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SUMMARY:

The effects of heating, on an aqueous gramicidin A lysolecithin system, were examined by carbon-13 nuclear magnetic resonance (^{13}C-NMR), circular dichroism (CD), and sodium-23 nuclear magnetic resonance (^{23}Na-NMR), and the results are collectively interpreted to indicate micellar-packaging of gramicidin channels and cation occupancy in the channel. ^{13}C-NMR of the gramicidin-lysolecithin system demonstrates a decrease in mobility of the micellar lipid on heating which is indicative of incorporation of gramicidin into the hydrophobic core of the micelle. A unique and reproducible CD spectrum is obtained for the heat incorporated state. Sodium-23 spin-lattice relaxation times (T_1) demonstrated sodium interaction to be dependent on heat incorporation. The T_1 identified interaction is blocked by silver ion which is known to block sodium transport through the channel in lipid bilayer studies. The temperature dependence of the sodium-23 line width defines an exchange process with an activation energy of 6.8 kcal/mole which is essentially the same as the activation energy reported for transport through the channel in lecithin bilayer studies, and the sodium exchange process is blocked by thallium ion which is also known to block sodium transport through the channel.

INTRODUCTION:

Gramicidin A, HCO-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$_{15}$-NH CH_2CH_2OH, is the first structurally described monovalent cation selective transmembrane channel (1-7). Heretofore, however, it has not been possible to apply the spectroscopic methods in the characterization of the gramicidin channel state. This is, in part, because gramicidin is polymorphic with its conformation being dependent on concentration, temperature and solvent system (8). Additionally, while it has been suggested that gramicidin at low concentration and temperature in trifluoroethanol is more relevant to the channel state (8), studies of cation binding in trifluoroethanol show Ca^{+2} to be strongly bound and Na^+ and K^+ to exhibit little or no

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evidence of interaction. On the other hand, in lipid bilayer transport studies, Ca^{+2} is neither transported nor bound to the channel (9) whereas Na^{+} and K^{+} are transported through the channel at rates of 10^{7} ions/sec (5). Accordingly, a primary objective of studies on the gramicidin channel becomes the development of an approach which will allow for characterization of the channel by spectroscopic methods, for example, to determine the circular dichroism pattern of the channel, to determine if there is cation occupancy in the channel, and, if so, to determine cation exchange with the channel. This information is essential to the complete characterization of this model for selective ion transport across biomembranes. The present communication reports circular dichroism (CD), carbon-13 magnetic resonance (^{13}C-NMR), and sodium-23 magnetic resonance (^{23}Na-NMR) data on the same sequentially treated samples of gramicidin A and lysolecithin and derives sodium exchange data and a CD spectrum which are argued to be representative of the channel state. The exchange data are particularly relevant to the energy profile for permeation through the channel and the fundamental question of whether the low dielectric constant of the lipid imposes a repulsive positive image force.

MATERIALS AND METHODS:

Egg yolk L-α-lysolecithin with mostly palmitic and stearic acid in position 1 was purchased from Sigma Chemical Company, St. Louis, Missouri and used without further purification. Micelles were formed by dispersing lysolecithin in deuterated water (99.71% D_2O) and sonicating for 3 minutes at power 3 in a Branson cell-disruptor model W-225R equipped with a cup horn accessory. Weighed amounts of gramicidin A, such that the potential concentrations of dimeric channels would be 1.3 mM, were added to the micelles in fixed volume of 10 mM NaCl in D_2O in order to obtain a 10:1 molar ratio of phospholipid to gramicidin A. The suspension was then shaken with a Vortex mixer for several minutes and sonicated for 6 minutes at power 4. The sample was incubated in a thermostatted bath at about 45°C for 10 minutes; and the spectroscopic studies were made. Then the same sample was heated at 68°C for 8 to 15 hours and the spectroscopic studies were repeated.

Circular Dichroism Measurements: CD spectra were obtained with a Cary 60 spectropolarimeter equipped with a Model 6001 circular dichroism attachment using 0.1 to 0.2 mm path length cells. Samples were diluted with distilled water and run at room temperature using the 0.1 or 0.04 degree scales.

^{13}C-NMR Measurements: Spectra were recorded on a JEOL PFT-100 spectrometer, operating at 25.15 MHz and equipped with a JEOL VT-3B variable temperature unit. With broad band proton noise decoupling, 30,000 to 50,000 transients were accumulated to obtain spectra using a pulse width of 20 μsec for a 90° magnetization vector and a repetition time of 1 second.

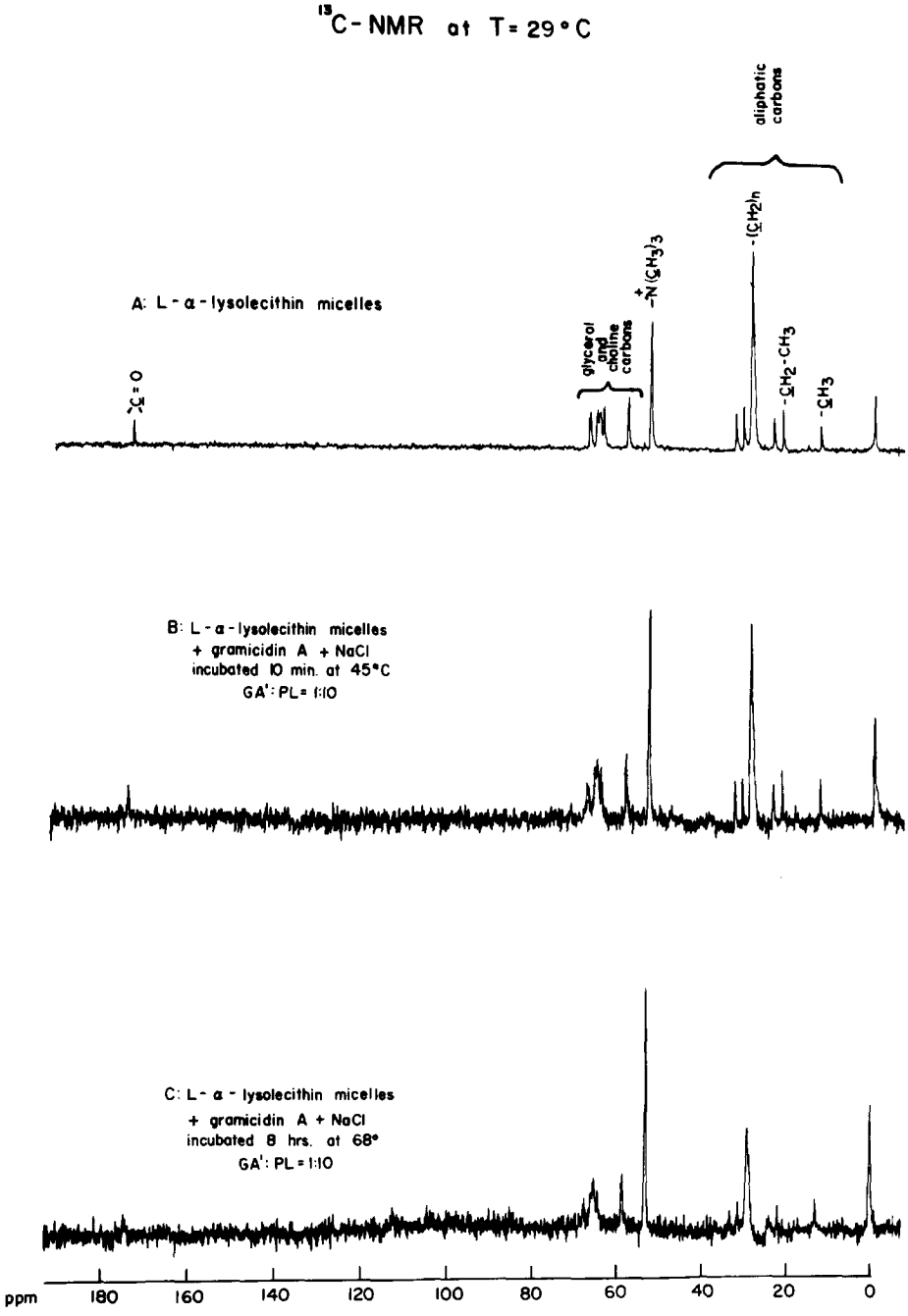

FIGURE 1: Carbon-13 magnetic resonance spectra at 25.15 MH of L-α-lysolecithin at 29°C. A. L-α-lysolecithin micelles; B. L-α-lysolecithin micelles which had been incubated at 45°C for 10 min. with gramicidin A at a molar ratio, phospholipid to gramicidin A, of 10:1; C.

^{23}Na-NMR Measurements: ^{23}Na-NMR spectra were obtained operating at 26.3 MHz on a JEOL FX-100 spectrometer equipped with a multinuclear probe. Spin-lattice relaxation times (T_1) for ^{23}Na were measured using the inversion recovery method (180^o-τ-90^o) with τ varying from 0.5 to 100 msec. At least 7 data points were used to calculate each value of T_1 in the least square method employing a Texas 980-B computer system.

In order to obtain parameters for sodium exchange with the channels, a lower, 1 mM, concentration of NaCl was used with a 40:1 phosphilipid to gramicidin A ratio and the sample was run at different temperatures maintained by a JEOL VT-3B variable temperature unit. Line widths at the half signal height were measured and after subtracting the line width of 28 Hz at half height obtained in the absence of gramicidin, the logarithms of the values were plotted against the inverse temperature in degrees Kelvin.

RESULTS AND DISCUSSION:

The ^{13}C-NMR spectrum of L-α-lysolecithin micelles in D_2O is given in Figure 1A with the resonance assignments indicated. The most intense resonance is that of the aliphatic $(\underline{C}H_2)_n$ carbons within the hydrophobic core of the micelle. The next most intense resonance is that of the most external choline methyl carbons. On addition of gramicidin A with short heating at 45^oC for 10 min, the choline methyl and $(\underline{C}H_2)_n$ peaks have approximately the same height (see Figure 1B). But on heating further at 68^oC for 8 hrs the intensities of the aliphatic carbon resonances are greatly reduced when compared to the choline methyl resonance. This indicates a relative decrease in the mobility of the hydrophobic core of the micelle as might be expected on heat-driven incorporation of a channel-containing, but outwardly hydrophobic, polypeptide structure.

Figure 2 contains corresponding circular dichroism spectra. With gramicidin A added dry to the micelles and with sonication but without heating, there is no detectable CD spectrum. On heating 10 min at 45^oC curve a is obtained with a negative 230 nm band and a positive 190 nm band. With further heating the CD spectrum converts to a new type of pattern dominated by a positive 220 nm

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L-α-lysolecithin micelles which had been incubated at 68^oC for 8 hours with gramicidin A at a molar ratio, phospholipid to gramicidin A, of 10:1. Compare curves A and C and note the relative loss of intensity of the aliphatic carbons. This reflects loss of mobility in the lipid core of the micelle.

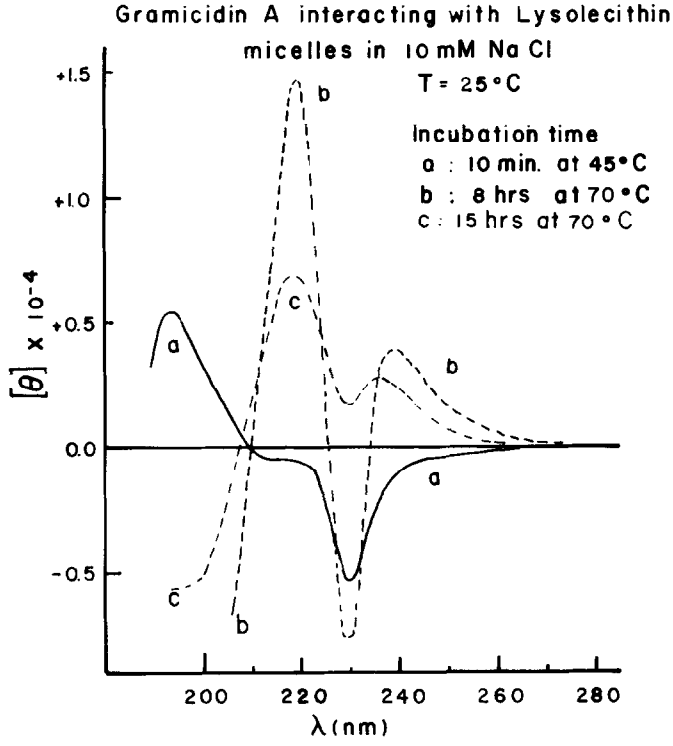
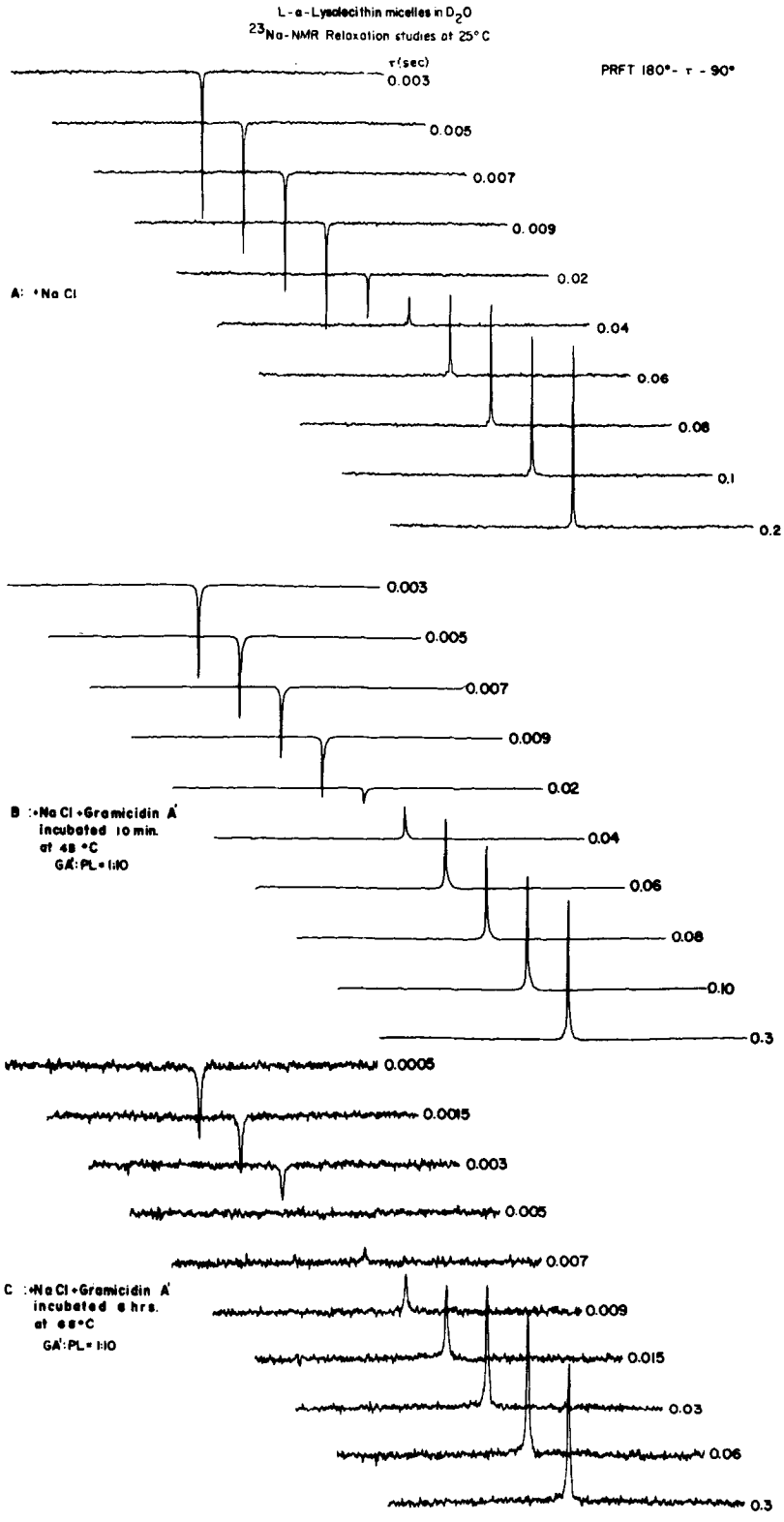


FIGURE 2: Circular dichroism data following the heat incorporation of gramicidin A into L-α-lysolecithin micelles. Curves a and b are spectra of the same samples shown in Figure 1 B and C, respectively. Curve c is the result of prolonged heating.

peak and by negative ellipticity below 205 nm. The intensity of the remaining negative 230 nm ellipticity band is quite variable but commonly is less intense as seen in curve c obtained on extended heating. The solution state which is most similar to curve c is that of hydrogenated gramicidin A in trifluoroethanol

FIGURE 3: Sodium-23 magnetic resonance longitudinal relaxation studies at 25°C of A. NaCl with L-α-lysolecithin micelles; B. the same sample as in Figure 1B and Figure 2a. C. The same sample as in Figure 1C and Figure 2b. In A the T_1 is 50 msec in B it is 45 msec and in C it is 10 msec. When the sample is incorporated such that the hydrophobic core of the micelle is perturbed there is evidence of interaction with sodium. The concentration of NaCl was 10 mM.


L-α-Lysolecithin micelles in D_2O
^{23}Na-NMR Relaxation studies at 25°C
τ(sec)
PRFT 180°- τ - 90°
0.003
0.005
0.007
0.009
0.02
A: +Na Cl
0.04
0.06
0.08
0.1
0.2
0.003
0.005
0.007
0.009
0.02
B :+Na Cl +Gramicidin A'
incubated 10 min.
at 48 °C
GA':PL= 1:10
0.04
0.06
0.08
0.10
0.3
0.0005
0.0015
0.003
0.005
0.007
C :+Na Cl +Gramicidin A'
incubated 6 hrs.
at 48°C
GA':PL= 1:10
0.009
0.015
0.03
0.06
0.3

at low concentration (8) with positive ellipticity in the 220 nm range and negative ellipticity below 205 nm. The interesting reciprocal behavior of the negative 230 nm peak and of part of the positive intensity of the 220 nm peak, seen on comparing curves b and c, may be due to tryptophan excitation resonance interactions as there is an intense absorption band near 225 nm.

The sodium-23 spin-lattice (longitudinal) relaxation studies are shown in Figure 3 where in A the value of T_1 for micelles alone is found to be 50 msec. On addition of gramicidin A with heating 10 min at 45°C, i.e. for the same sample as in Figure 1B and curve a of Figure 2, the T_1 is 45 msec. On heating for 8 hrs at 68°C, i.e. for the same sample as Figure 1C and curve b of Figure 2, the T_1 is found to decrease to 10 msec (see Figure 3C). This decrease in T_1 is lost on addition of 10 mM $AgNO_3$ to a 10 mM $NaNO_3$ study where the 10 msec relaxation time is raised to 41 msec. The silver ion, which inhibits sodium ion transport through the channel (10), competitively inhibits the interaction of Na^+ with gramicidin A once it is in a state which perturbs the hydrophobic core of the micelle.

On reducing NaCl concentration to 1 mM, the line width of the sodium-23 resonance is found to greatly broaden, indicative of exchange with a binding site on the gramicidin A. This observation allows characterization of an energy of activation for the exchange process (11). In Figure 4 is a plot of the line broadening as a function of T^{-1} (°K). From the slope, the activation energy for the exchange process is approximated to be 6.8 kcal/mole. The activation energy for transport through the gramicidin A channel in black lipid membranes depends on the lipid and has been reported to vary from about 5 kcal/mole for glyceryl monooleate-decane membranes (3) to 7.3 kcal/mole for dioleoyl lecithin membranes (12). In the present work is characterized an exchange process in gramicidin A-doped lysolecithin lipid structures with an activation energy in the same range as observed for transport through the gramicidin A-doped lipid bilayers. A further check, as to whether this corresponds to interaction with the channel state, can be sought with the thallous ion, Tl^+, which blocks Na^+

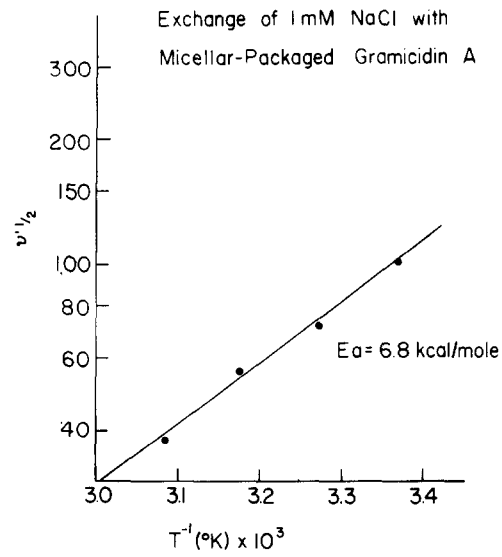


FIGURE 4: Line width of the sodium-23 nuclear magnetic resonance line of 1mM NaCl as a function of temperature. The broadened resonance is due to interaction with gramicidin and the 6.8 kcal/mole barrier is the energy of activation for sodium exchange with a gramicidin binding site.

transport through the channel (13,14). Addition of 10 mM thallium chloride results in the complete narrowing of the sodium resonance line and, thereby, completely removes evidence of interaction of $^{23}Na^+$ with the micelle incorporated gramicidin A.

Based on the criteria of perturbing the hydrophobic core, of producing a unique CD spectrum, of exhibiting an interaction with sodium ion as evidenced by changes in spin-lattice relaxation times that are reversed by silver ion which blocks the channel, of exhibiting an exchange process with an activation energy which is similar to that observed for transport through the channel and which is blocked by Tl^+ which blocks Na^+ transport through the channel, it is proposed that the heat treatment is effectively incorporating gramicidin A channels into lysolecithin lipid structures.

That the activation energy for the exchange process is similar to that of transport through the channel has significant implications with respect to the energy profile for movement of ions through the channel. The exchange is with a channel binding site and the energy barrier for reaching that binding site from the solution is essentially the same as the highest barrier for movement of Na^+ completely through the channel. With the symmetrical channel, being formed from the head to head hydrogen bonding of two molecules (1,2), this requires that the dominating energy barrier is not at the center of the membrane where the low dielectric constant of the lipid would have its most pronounced effect (14,15). This is consistent with the realization that nearly the complete hydration energy can be achieved by the lateral peptide carbonyl coordinations plus the binding of water preceding and following the cation through the channel (16,17).

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