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INFRARED SPECTRA OF THE GRAMICIDIN A TRANSMEMBRANE CHANNEL: THE SINGLE-STRANDED-β^6-HELIX

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IR spectra are reported for preparations of Gramicidin A and malonyl Gramicidin A incorporated as the channel state in phospholipid structures. In this preparation Gramicidin A has already been shown to be unequivocally in the single-stranded β-helical conformation. The result is an amide I frequency of 1633 ± 1 cm^{-1}. This demonstrates that the single-stranded β-helix has an amide I frequency that has previously been considered to be diagnostic of antiparallel double-stranded β-helix and of β-sheet structures.

Based on different approaches, a number of laboratories actively engaged in the study of the Gramicidin A transmembrane channel have concluded that the structure of the channel is the head to head dimerized single-stranded β-helix (1-12). One approach has been to study the conductance in planar lipid bilayer membranes that was induced by Gramicidin A (GA) and a series of its derivatives and analogs (1-6). A second approach has involved the application of physical methods such as nuclear magnetic resonance and circular dichroism to the study of membrane suspensions containing Gramicidin A in the channel state (7-12). In contrast to the conclusion from these varied approaches, studies utilizing infrared spectroscopy as the basic methodology have led to the conclusion that the dominant channel state is an antiparallel double-stranded β-helix (13,14). The basis is a theoretically calculated amide I frequency for a series of single- and double-stranded β-helical conformations of Gramicidin A (15) using the approach of Chirgadze and Nevskaya (16-19). The assumption was made (15) that the magnitude, direction and location of the transition dipole moment for the double-stranded β-helices would be the same as for the β-pleated sheet structures and that for the single-stranded β-helices would be the same as for the α-helix. The result was that the amide I

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frequency of the antiparallel double-stranded $\beta^{5\cdot6}$ helix was calculated to be at 1634 cm^{-1} and the head to head single-stranded $\beta^{6\cdot3}$-helix was calculated to be at 1656 cm^{-1}. It is further being assumed in the literature (19) that an amide I band at 1633 cm^{-1} is conclusive evidence for the absence of single-stranded β-helices.

Recently a simple, unequivocal determination of the structure of Gramicidin A, properly incorporated into phospholipid suspensions as the channel state, has been achieved (20). The approach was to synthesize a series of Gramicidin A molecules. In each synthesis only one carbonyl carbon was ^{13}C-enriched at greater than 90%. The series of structures were: (1-^{13}C) L•Val^{1} GA, (1-^{13}C) L•Ala^{3} GA, (1-^{13}C) L•Ala^{5} GA, (1-^{13}C) L•Val^{7} GA, (1-^{13}C) D•Val^{8} GA, (1-^{13}C) L•Trp^{9} GA, (1-^{13}C) L•Trp^{11} GA, (1-^{13}C) L•Trp^{13} GA, (1-^{13}C) D•Leu^{14} GA, and (1-^{13}C) L•Trp^{15} GA. The carbon-13 nuclear magnetic resonance (cmr) spectra were determined for each of these molecules incorporated into phospholipid as the channel state and the cmr spectra were then obtained in the presence of ions which are transported by, and are bound at sites within, the channel. The presence of an ion induced carbonyl carbon chemical shift was used to locate the ion binding site. Using the argument that helically equivalent carbonyls equally proximal to the binding site should show similar ion-induced carbonyl carbon chemical shifts, it was possible to show that the assumption of antiparallel (and parallel) double stranded β-helices resulted in an unacceptable contradiction. For example, for antiparallel double-stranded β-helices, carbonyls of residues 3 and 5 are in the same segment of structure as the helically equivalent carbonyls of residues 11 and 13, yet carbonyl carbons 3 and 5 showed no ion-induced chemical shifts while carbonyl carbons 11 and 13 showed large ion-induced chemical shifts. This contradiction requires that the antiparallel double stranded β-helices be eliminated as possible structures. Considerations utilizing all of the data demonstrated the head to head dimerized, left-handed, single-stranded β^{6}-helix to be the correct structure.

Having established the structure of the channel in the phospholipid suspension to be the single-stranded β-helix, in this manuscript we report infrared spectra on this system.

MATERIALS AND METHODS:

Malonyl-bis-desformyl Gramicidin A was prepared from commercially available Gramicidin (ICN Nutritional Biochemicals Corporation, Cleveland, OH) as previously described (21). The material was found to be pure by ^{13}C magnetic resonance and by circular dichroism. The naturally occurring formyl Gramicidin A (ICN) was lyophilized from a methanol-water mixture and was used without further purification. L-α-lysolecithin (Avanti Biochemicals, Birmingham, AL) was found to be free of any unsaturated fatty acid by ^{13}C magnetic resonance and was also used without further purification. Samples were prepared in solutions of 0.5 mM NaCl in D_2O, 99.87%D (Sci Graphics, Wayne, NJ). Both malonyl Gramicidin and natural Gramicidin were packaged into lysolecithin structures using the sonication and heat incubation procedure previously described (22). The progress of channel formation was checked by observing the circular dichroism spectrum of each sample after several hours incubation at 65 - 70°C. When the appropriate spectra were obtained (10,22), the samples were centrifuged and the supernatants removed. The ^{23}Na magnetic resonance spectrum of each sample was then observed in order to confirm that the ion was interacting with the channel state (21,23). The concentration of channels in each sample was determined by observing the ultraviolet absorbance of a known volume of supernatant resuspended in methanol. For the malonyl and for the natural Gramicidin samples, the channel concentration was found to be 2.4 mM and 5.9 mM respectively. Phospholipid concentrations in both samples was 0.1 M. The circular dichroism spectra for both incorporations are given in Figure 1.

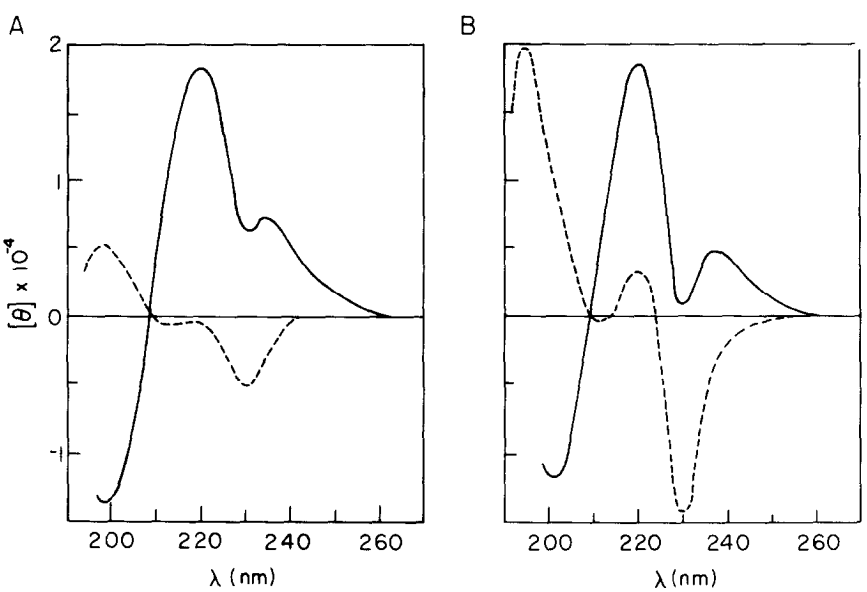

FIGURE 1: Circular dichroism spectra of Gramicidin A and malonyl Gramicidin A in lysolecithin phospholipid bilayer membranes. The positive 220nm peak and the low magnitude of the negative peak near 230nm are indicative of the channel state. On initial association with lysolecithin micelles before any incorporation has occurred, the dashed curve in A is obtained. An intermediate state on the way to the channel state is given as the dashed curve in B.

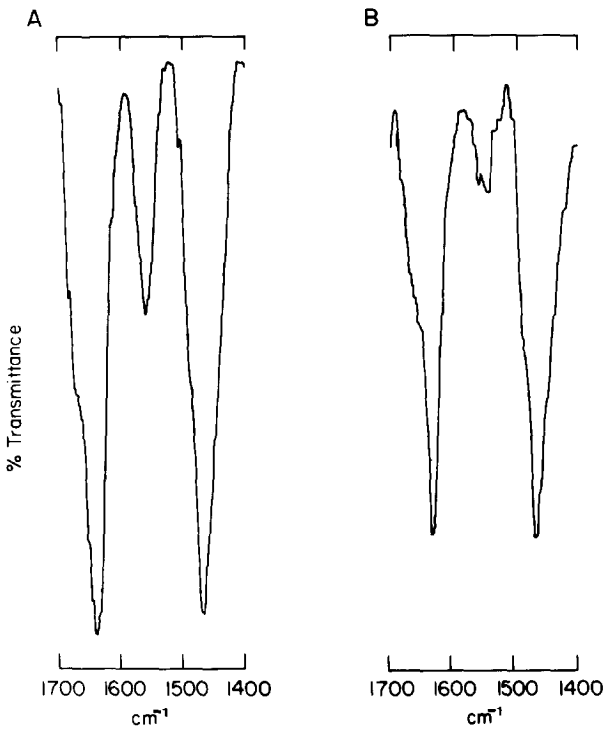

FIGURE 2: IR spectra A. of Gramicidin A and B. of malonyl Gramicidin A in the channel state in phospholipid bilayer membranes. The amide I band is at 1633 ± 1 cm^{-1}.

For infrared spectroscopic characterization 0.1 ml of suspension was evenly applied to a Beckman KRS-5 IR window and allowed to dry overnight. The spectra were obtained on a Beckman IR-12 spectrophotometer at a scanning speed of 8 cm^{-1} and a period of 2. The wavenumber calibration was carefully checked using the polystyrene resonances at 1601 cm^{-1} and 1027.7 cm^{-1}.

RESULTS AND DISCUSSION:

The IR spectra in the 1400 to 1700 cm^{-1} range are plotted in Figure 2 for both Gramicidin A and for the malonyl covalent dimer of Gramicidin A. In both cases the result obtained from numerous spectra is an amide I resonance at 1633 ± 1 cm^{-1}.

The experimental value of 1633 ± 1 cm^{-1} for the amide I frequency of the Gramicidin A transmembrane channel in the phospholipid incorporation has previously been considered to be conclusive evidence for the dominance of the antiparallel double-stranded β-helix (13,14) and also to be indicative of the absence of single-stranded β-helix (19). Accordingly, it is pertinent to review more thoroughly the most specific data which excludes the antiparallel

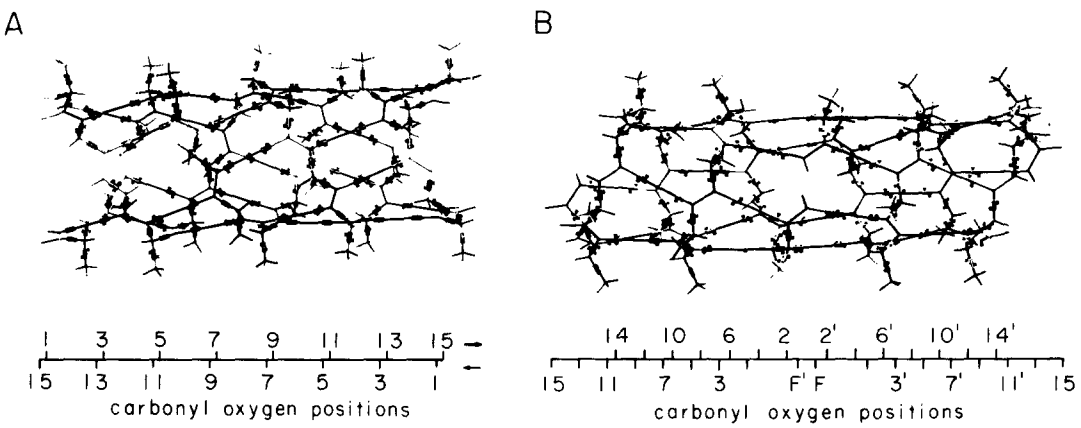

FIGURE 3: A. Wire model of an antiparallel double-stranded β-helix with a scale indicating the positions of the L-residue carbonyls for each chain. B. Wire model of single-stranded β-helix with a scale indicating the positions of the L- and D-residue carbonyls for each chain.

double-stranded structure from being the structure of Gramicidin A in the phospholipid structure under examination here.

In Figure 3A is a wire model of an antiparallel double-stranded β-helix and below the structure is a scale that gives the relative positions of the L-residue carbonyls for the two chains. It is seen, for example, that carbonyls of L-residues 11 and 13 of one chain occur in the same section of the channel as the carbonyls of L-residues 3 and 5. These are helically equivalent carbonyls that occur in the same segment of channel and they would be expected to exhibit similar responses when equally proximal to an ion. What has been found (11), however, is that for both Na^+ and Tl^+ large chemical shifts are observed in the carbon-13 nuclear magnetic resonance spectra for the carbonyls of L-residues 11 and 13 and smaller shifts are exhibited by residues 9 and 15 when the carbonyl carbons of these residues are ^{13}C-enriched. On the other hand the carbonyl carbons of L-residues 1, 3, 5 and 7 exhibited no ion-induced carbonyl carbon chemical shifts when these carbonyl carbons were ^{13}C-enriched (20). Accordingly the antiparallel double-stranded β-helix cannot be a significant structure in the preparation examined by infrared spectroscopy in the present manuscript. On the other hand, as shown in Figure 3B for the head to head dimerized single-stranded β-helix, this is readily explained by two

binding sites, one just inside of each entry to the channel. In addition, an ion-induced carbonyl carbon chemical shift exhibited by D-residue-14 and the absence of one exhibited by D-residue 8 (12) allow dismissal of the parallel double-stranded β-helix and demonstrate that the single-stranded β-helix is left-handed. This is further confirmed by the demonstration that divalent cations such as Ba^{+2} which decrease monovalent cation currents but are not transported (24) bring about ion-induced carbonyl carbon chemical shifts for residues 11, 13, 14 and 15 but do not cause any chemical shift in the carbonyl carbon resonances of residues 1 and 8 (12,20,25). Accordingly, the structure that forms on incorporation of Gramicidin A into phospholipid structures to give the CD pattern in Figure 1 cannot be a double-stranded β-helix but rather is the single-stranded β-helix. Additionally, the CD pattern of Figure 1 is found for Gramicidin A and its analogues and derivatives which do form conducting channels but is not found for those which do not (9,10). Characteristically, those derivatives which do not form conducting channels have an intense negative band at 230nm as in the dashed curves of Figure 1.

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TABLE 1

Concentration of RNA isolated from the mammary explants of adrenalectomized virgin rats.

Culture conditions	RNA; μg per mg wet tissue
t_o	0.46 ± 0.025
I	1.03 ± 0.049
IP	0.91 ± 0.064
IFP	0.83 ± 0.060

The experimental details were the same as those described in the legends to Figs. 2 and 3. Each value represents the mean ± S.E.M. for 3 groups of rats, with 3 animals in each group.

A much smaller dependence of casein gene expression on exogenous glucocorticoid in mammary explants from intact pregnant rats was reported previously (3). This might be explained by the fact that the protocol used did not take into account fully the observation that isolated mammary tissue from pregnant rats retains about 30% of the hydrocortisone to which it had been exposed 6 days previously (5). This explanation might also apply to the protocol (3) in which tissue from pregnant rats, adrenalectomized only 2 days before sacrifice, was used. In both cases, it is likely that the tissue had retained some endogenous glucocorticoid during culture.

It appears that casein gene expression in isolated rat mammary tissue is highly, and perhaps entirely, dependent on glucocorticoid. This dependency does not reflect a loss of cell viability in the absence of the steroid.

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