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Synthesis, Characterization, and Black Lipid Membrane Studies of [7-L-Alanine]gramicidin A†

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ABSTRACT: With a view to study the relevance of side-chain orientation in the transport of cations through a gramicidin transmembrane channel and to identify an analogue with favorable characteristics, [L-Ala^7]gramicidin A was synthesized, purified, verified, and characterized by high-performance liquid chromatography, by carbon-13 and proton magnetic resonance spectra, and by circular dichroism spectra in methanol. Complete incorporation as the channel state was achieved when packaged in lysolecithin-containing lipid bilayers. The single-channel conductance data in diphytanoyllecithin/*n*-decane membranes are presented along with those of synthetic gramicidin A (GA). [L-Ala^7]GA exhibits the highest most probable single-channel conductance so far reported for an analogue occurring at 28 pS as compared to 21 pS for GA under similar conditions. Also, a dramatic reduction in the dispersity of conducting states is observed with about 76% of the events falling in a narrow 1.75-pS conductance window as compared to about 31% of the events for GA under identical conditions. Thus, with the above characteristics, [L-Ala^7]GA appears to be a very good candidate for a thorough study of ionic mechanism. The present results indicate that elements intrinsic to the channel proper are rate-limiting for GA and that there is no interfacial polarization or diffusion-controlled association at 1 M KCl and a 100-mV applied potential.

Gramicidin, a pentadecapeptide, isolated from a strain of *Bacillus brevis* (Hotchkiss & Dubos, 1940) was shown to be a mixture of gramicidins A, B, and C (Gregory & Craig, 1948) with alternating L- and D-amino acids differing in position 11, with Trp, Phe, or Tyr (Gross & Witkop, 1965), respectively, in the ratio of 72:9:19 (Glickson et al., 1972). Each in turn had either Val or Ile in position 1. The structure of [Val^1]-gramicidin A was determined to be (Sarges & Witkop, 1965a,b)

H-C(O)-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}-$NHCH_2CH_2OH$

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Gramicidin forms transmembrane channels in lipid bilayers (Mueller & Rudin, 1967; Hladky & Haydon, 1972). The kinetic studies have shown that two molecules are necessary to form such a channel (Tosteson et al., 1968; Bamberg & Läuger, 1973). The channel structure (Urry, 1971; Urry et al., 1971) that is now widely accepted is that two molecules of gramicidin come together at their N-termini (formyl ends) to form the channel with a pore diameter of $\simeq$4 Å and a length of $\simeq$26 Å. The molecular structure of each monomer is such that they form β-helices with 6.3 residues per turn and with a helical pitch of 4.8 Å. The two single-stranded $\beta^{6.3}_{3.3}$ helices, as they are called, are stabilized by 6 intermolecular and 22 intramolecular hydrogen bonds. The structure was further confirmed by the synthetic analogues (*N*-pyromellityl and *O*-pyromellityl) of gramicidin (Bamberg et al., 1977). The alternate double-stranded helical model for the gramicidin channel was dropped after ^{19}F and ^{13}C nuclear magnetic resonance studies on gramicidin analogues in the lipid-incorporated state (Weinstein et al., 1979, 1980).

X-ray diffraction studies on K^+ and Cs^+ complexes of gramicidin have indicated the presence of two ion binding sites in the channel (Koeppe et al., 1979). After lipid incorporation of the channel state of several synthetic gramicidin analogues having carbonyl carbon enriched (>90%) amino acids substituted one at a time along the peptide chain (e.g., [[1-^{13}C]Trp^{15}]GA, [[1-^{13}C]Trp^{13}]GA, and so on), mapping of the carbonyl chemical shifts on interaction with sodium ion was carried out. The results clearly demonstrated the presence and exact location of the two symmetrically situated ion-binding sites between the Trp^{11} and Trp^{13} positions, one on each end of the dimeric gramicidin channel (Urry et al., 1982a). Further studies on [D-Val^8]- and [D-Leu^{14}]GA analogues are also consistent with the left-handedness of the β-helical structure of the channel (Urry et al., 1982b), though a right-handed helical sense has recently been argued in sodium dodecyl sulfate micelles by using NMR (Arseniev et al., 1985). The inner wall of the channel is lined by the polar peptide carbonyls whereas the outside has all the hydrophobic amino acid side chains, rendering the molecule very suitable for incorporation into the hydrophobic environment of the lipid bilayer system. One of the interests in studying the gramicidin channel lies in its ion selectivity, which is similar to that of physiological channels. Only monovalent cations (Myers & Haydon, 1972) but not divalent cations (Hladky & Haydon, 1972) can pass through the channel. In order to explain small cation translocation through the channel with an approximate 4-Å pore diameter, Urry (1973) has proposed a peptide libration mechanism by which the peptide carbonyls in the β-helical structure would librate inward, decreasing the channel radius, resulting in a better coordination with the cations and thus aiding in their transport through the channel.

The gramicidin channel in the black lipid membrane (BLM) has generally a well-defined conductance that remains stable during the lifetime of the channel. A typical histogram representing the frequency of occurrence of the channels and their conductance shows the presence of several widely dispersed lower conducting states. The reason for this was originally considered to be due to the inhomogeneity of the natural gramicidin as indicated above. But when a wholly synthetic [Val^1]GA and a high-performance liquid chromatography (HPLC) purified [Val^1]GA from a natural gramicidin mixture were examined in BLM studies, identical histograms were obtained with the same dispersity of single-channel conductance states (Prasad et al., 1982a). This ruled out the possibility that the dispersity was due to the heterogeneity of natural gramicidin. The dispersity of the conducting states is also not due to the possibility of various other channel structures such as head to tail or tail to tail association of the two monomers (Urry 1971; Bradley et al., 1978) or due to the parallel and antiparallel double-stranded structure (Weinstein et al., 1979, 1980), because it is well established now that more than 90% of the gramicidin channels are due to single-stranded head to head associated β-helices both in planar bilayers (Bamberg et al., 1977; Apel et al., 1977) and in suspensions of bilayer membranes (Weinstein et al., 1979; Urry et al., 1983). Channels having different radii and variation or defects in hydrogen bonding were also suggested, but then it is difficult to explain the observed switching between "mini" and regular channels without the main conducting channel being turned off (Busath & Szabo, 1981).

An alternate suggestion for the dispersity of the single-channel conductances was put forward (Urry et al., 1981) which says that the energetics of the libration of carbonyl carbons interacting with the cations would be influenced by the orientation of the different side chains in the gramicidin channel structure. A stereo plot of the low-energy conformation of the head to head dimer is given in Figure 1A, and the channel view from the solution of one monomer is given in Figure 1B. It is evident from these conformations that there will be interactions between side chains of amino acid residue i with $i \pm 1$ and also with $i \pm 6$. By changing the bulkiness of the side chain of one amino acid, therefore, one could effectively alter the side-chain orientation of the other interacting amino acids, which in turn could affect the energetics of the libration of carbonyl carbons interacting with cations and hence change the magnitudes of the single-channel conductances.

In order to test this hypothesis, des-L-Val^7-D-Val^8-GA was synthesized. In this analogue, the restriction imposed on the Trp^{13} side chain due to the bulky side chain of L-Val^7 was removed. The $i \cdots i-6$ interaction would then be between the Trp^{13} and the less bulky Ala^5 side chains, and this could result in several more conformational states possible for this analogue and hence could result in more dispersity of the single-channel conductances. Actually, a very broad peak covering a 20-pS range from 5 to 25 pS centered at 17 pS was obtained, whereas under similar experimental conditions the most probable peak for GA was centered at 26 pS covering a 4-pS range (Urry et al., 1984a). A dispersity of single-channel conductances for this analogue was also reported by others (Ovchinnikov & Ivanov, 1977). A second analogue, [L-Leu^5]GA was synthesized with the objective of reducing the freedom of the side-chain orientations of Trp^{11}, which would then lead to reduced dispersity of the single-channel conductances. As expected, more than 68% of the events occurred under one main peak for [L-Leu^5]GA whereas only 49% of the events occurred under one main peak for HPLC-purified synthetic GA (Urry et al., 1984b) under similar conditions. In this paper we report the synthesis, characterization by proton and carbon-13 magnetic resonance spectra (^{1}H NMR and ^{13}C NMR), phospholipid incorporation, HPLC, and single-channel current data of [L-Ala^7]GA, that was planned on the above hypothesis to assess the effect of the bulky L-Val^7 side-chain interaction with the Trp^{13}. This gives the Trp^{13} side chain freedom to assume additional energetically favorable orientations.

Materials and Methods

Synthesis. The synthesis of [L-Ala^7]GA was carried out by the solid-phase method (Merrifield, 1963) with a few modifications as reported earlier (Prasad et al., 1982a; Urry et al., 1983). The *tert*-butyloxycarbonyl (Boc) group was used for N$^\alpha$-protection, and its removal was effected with 33%

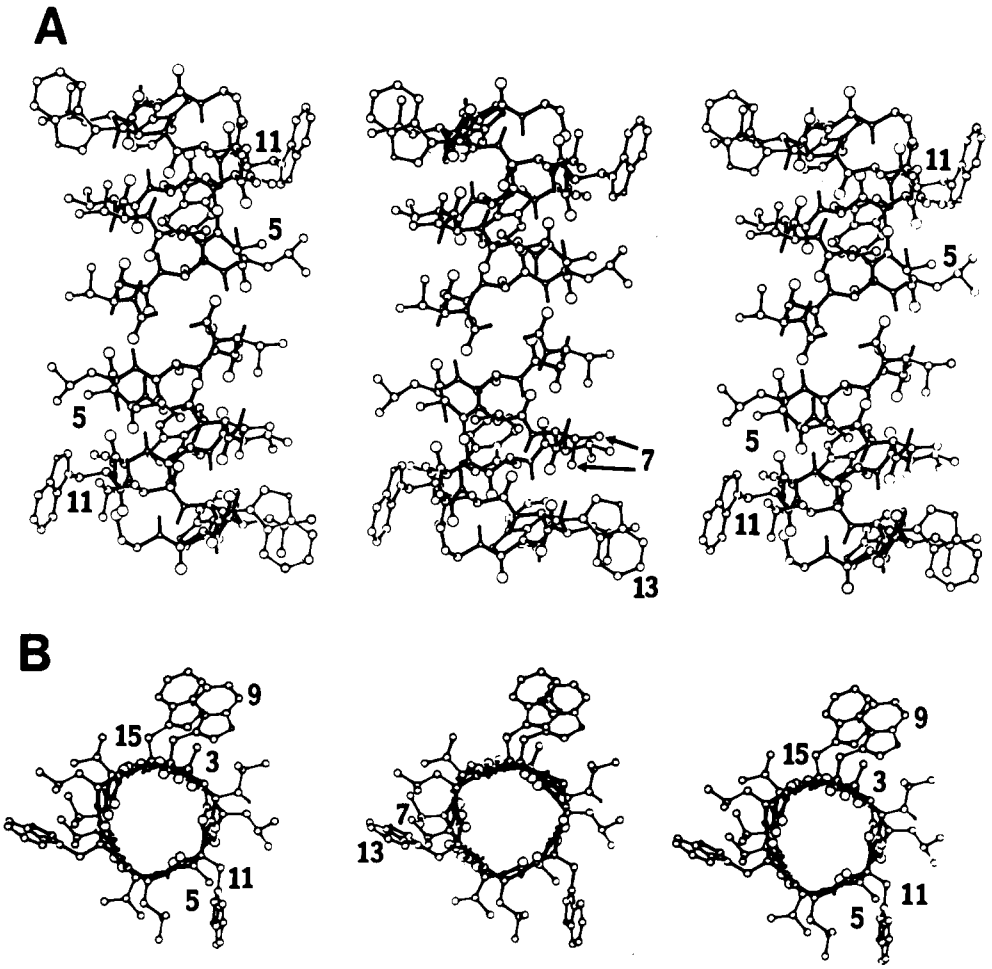

FIGURE 1: Stereoviews of the in vacuo most energetically favorable conformations of the gramicidin transmembrane channel. Three views are plotted in order to allow for either near (cross-eye) or distance (wall-eye) viewing. For wall-eye viewing, place a piece of paper over the left-hand structure and merge the right-hand pair. For cross-eye viewing, cover the right-hand structure and use the left-hand pair. Note the $i \cdots i - 6$ interaction of Trp^{13} and Val^{7} side chains. Adapted with permission from Venkatachalam & Urry (1983).

TFA/CH_2Cl_2 containing 5% 1,2-ethanedithiol and 6% anisole (5 and 25 min). The neutralization step was carried out with 5% diisopropylethylamine in CH_2Cl_2 (2 × 6 min). The coupling reaction was carried out with 2.5 equiv each of Boc-amino acid and dicyclohexylcarbodiimide in CH_2Cl_2. When needed, the coupling step was repeated in 50% DMF/CH_2Cl_2, and uncoupled chains were finally acetylated. After the synthesis of the pentadecapeptide was completed, the Boc peptide was removed from the resin support by ethanolamine/CH_3OH treatment (yield 91%) and deblocked with 50% TFA/$CHCl_3$ containing 5% 1,2-ethanedithiol and 6% anisole. After the solvent was removed, the peptide was passed through a column of AG 50W-X2 (H^+ form) resin whereby most of the acetylated products were eluted by methanol, and the desformylgramicidin analogue was eluted in 2 N methanolic ammonia solution (yield 87%). The peptide was formylated with formic acid/acetic anhydride and again passed through a cation-exchange column to remove any unreacted material (yield 61.5%, not taking into account the amount of recovered unreacted material). Purification was achieved by preparative thin-layer chromatography (TLC) using the $CHCl_3$–CH_3OH–CH_3COOH (85:15:3) solvent system, and the peptide was eluted from silica gel with 20% CH_3OH/acetone. LH-20 column chromatography was used to remove any silica gel picked up during the elution procedure from silica gel plates and also to effect further purification of the peptide. All the fractions that had shown a single spot on TLC were combined and lyophilyzed from methanol–water. A highly purified material in ≃15% yield was obtained. [L-Ala^7]GA gave one major peak on HPLC and the correct amino acid ratios.

^1H NMR and ^{13}C NMR Spectra. The carbon-13 nuclear magnetic resonance spectra were obtained on a JEOL PF-T-100 spectrometer equipped with a 10-mm probe operating at 25.15 MHz and 25 °C under conditions of internal deuterium lock and proton noise decoupling. A Varian HR-220 spectrometer was used to record the proton magnetic resonance spectra at 220 MHz with a 5-mm probe at 20 °C. Samples of the gramicidin were dissolved in [$^2H_6$]dimethyl sulfoxide (Merck Sharp & Dohme, Pointe Claire, Canada) at concentrations of 30 mM.

Phospholipid Packaging of the Synthetic [L-*Ala^7*]*GA.* A Cary 60 spectropolarimeter equipped with a Model 6001 circular dichroism (CD) attachment modified for high-frequency modulation was used to record the CD spectra of the synthetic gramicidin in methanol and in phospholipid bilayers. Concentrations of the peptide were verified by observing the ultraviolet (UV) spectra in methanol with $\epsilon = 22\,500$ L mol^{-1} cm^{-1} for gramicidin. [L-Ala^7]gramicidin was packaged into L-α-lysolecithin (Avanti Polar Lipids, Inc., Birmingham, AL) bilayers (Pasquali-Ronchetti et al., 1983; Killian et al., 1983) by the sonication and heat-incubation procedure previously described (Urry et al., 1979). The lipid to gramicidin ratio was 30:1. A small amount of NaCl (0.5 mM) was present in the sample during incubation, which was carried out for 40 h at 70 °C. The lysolecithin–gramicidin suspension was then

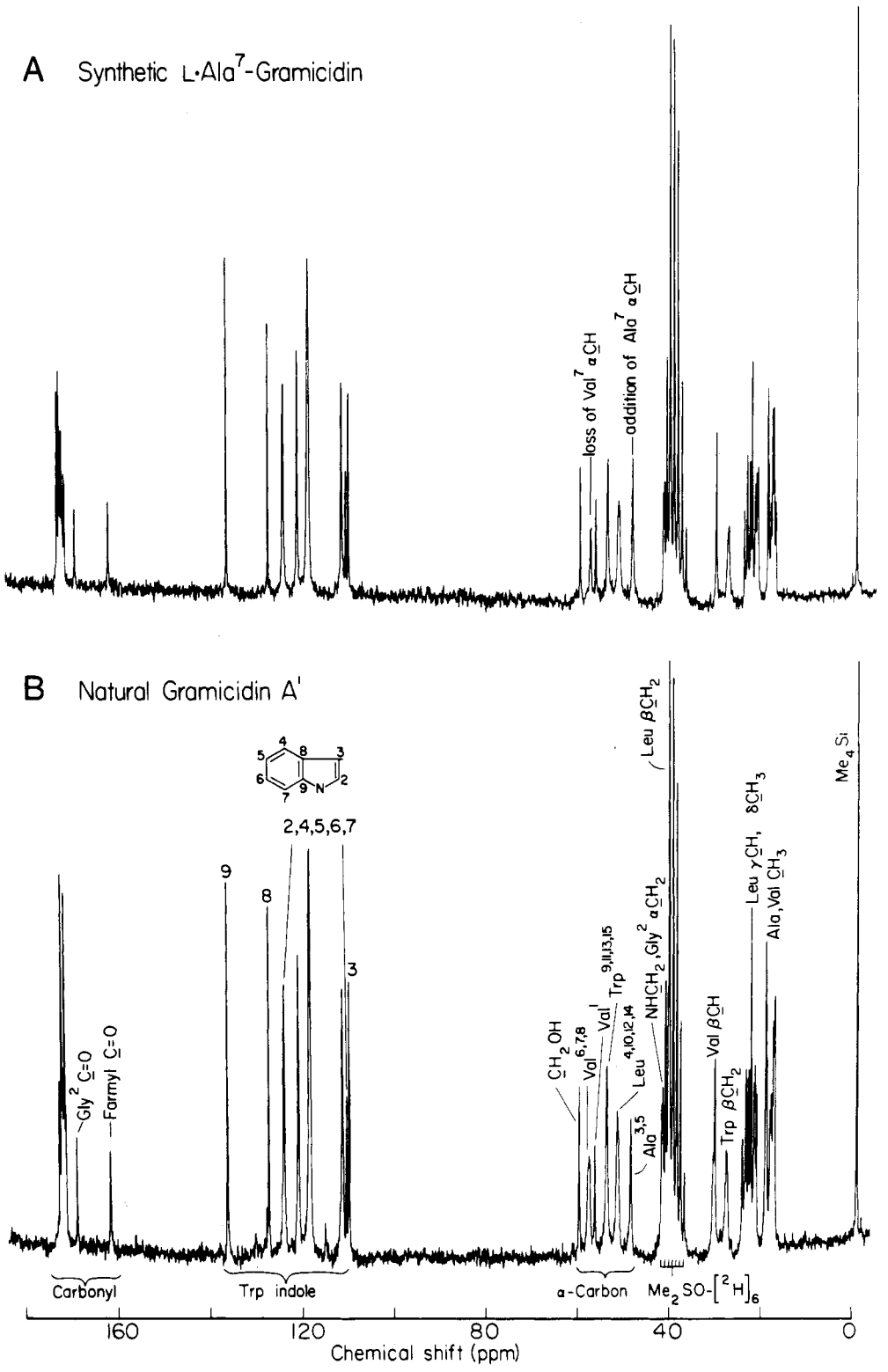

FIGURE 2: Carbon-13 magnetic resonance spectra at 25.15 MHz of (A) synthetic [L-Ala^7]GA and (B) a natural gramicidin mixture in [$^2H_6$]dimethyl sulfoxide at 25 °C.

centrifuged to remove any unincorporated peptide, and the supernatant was checked for completeness of channel formation by observing the CD spectrum and by measuring the sodium-23 chemical shift and line width at 30 °C.

HPLC Purification for BLM Studies. A sample of [L-Ala^7]GA was further purified by repeatedly passing the main peak of the peptide over an analytical Zorbax-ODS column (4.6 mm × 25 cm) with a 15% H_2O/CH_3OH solvent system on a Beckman Model 332 gradient liquid chromatrograph at room temperature with UV monitoring at 220 nm.

Single-Channel Conductance Measurements. Black lipid membranes were formed on a 0.6-mm diameter aperture separating two Teflon chambers, each filled with 7 mL of 1 M KCl solution as described previously (Bradley et al., 1977). A 2% solution of diphytanoyllecithin in *n*-decane (w/v) was used to form the membrane. Picomolar concentrations of HPLC-purified [L-Ala^7]GA were added to the bath from methanolic stock solution. Data were acquired only after

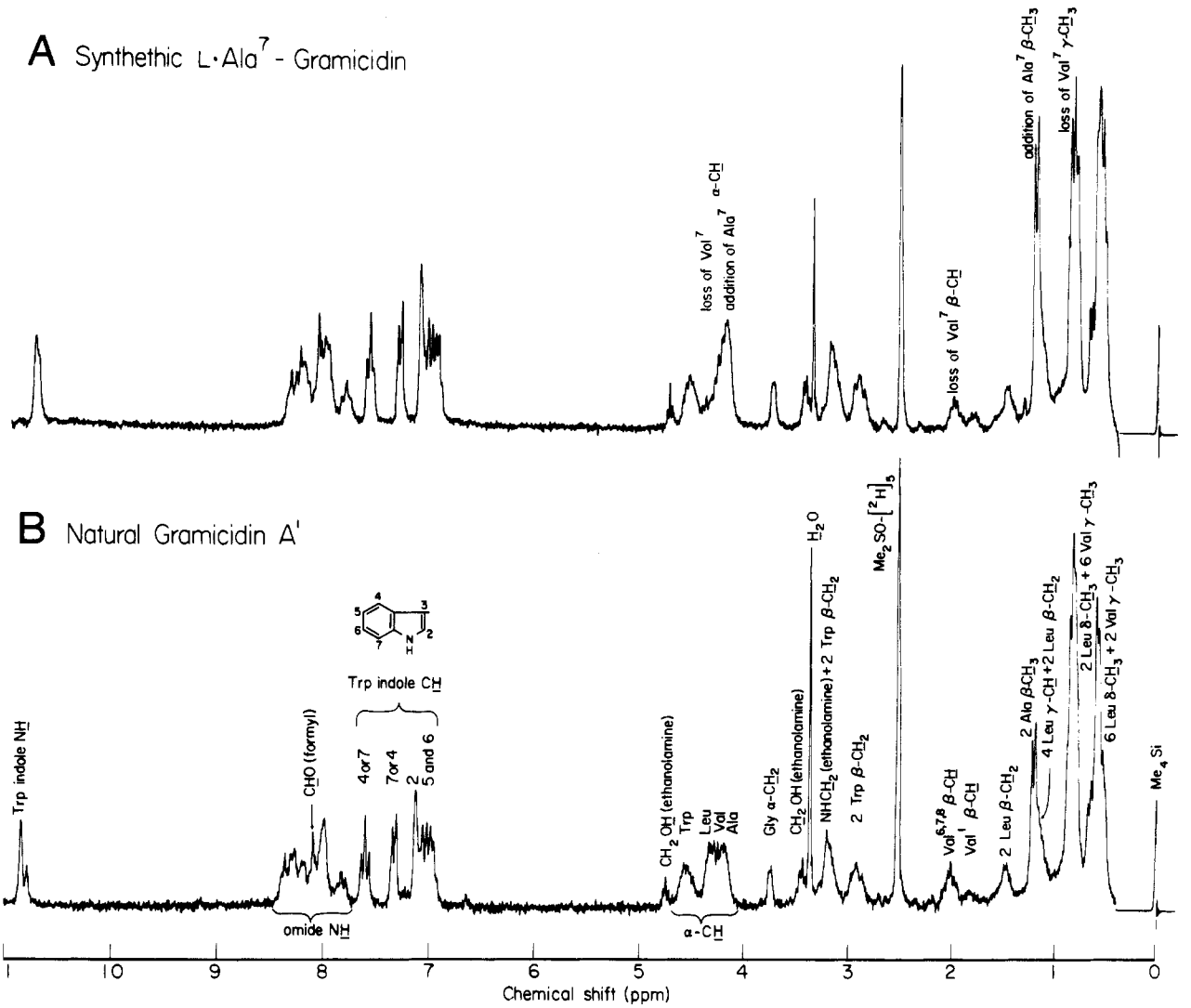

FIGURE 3: Proton magnetic resonance spectra at 220 MHz of (A) synthetic [L-Ala^7]GA and (B) a natural gramicidin mixture in [$^2H_6$]dimethyl sulfoxide at 20 °C.

membrane stability was achieved (~1 h). The temperature of the cell was maintained to within ±0.1 °C by means of a Peltier cell to give 26.8 °C for gramicidin A and 25.4 °C for the L-Ala^7 analogue. The electrical measurements were made with Ag–AgCl electrodes, and the applied voltage clamp was 100 mV. The cell and the voltage source are placed in a small shielded metal box mounted upon a vibration-free air-isolated Micro-g table. The collection of data and analysis of the single-channel currents are described in detail elsewhere (Urry et al., 1984c; Venkatachalam et al., 1984).

RESULTS

The synthesis of [L-Ala^7]GA was carried out by the solid-phase method as described under Materials and Methods. The yield of the peptide after removal from the resin was 91%, and the final yield of the very pure material was only 15%. This drop in yield may be due to the destruction of Trp side chains during repeated acid treatment (TFA treatment for Boc removal) and also due to the formation of truncated peptides. The use of a base-labile [(9-fluorenylmethyl)oxy]carbonyl group for N$^\alpha$-protection did not improve the yield to any considerable extent because of other problems involved (Prasad et al., 1982b). A major reduction in yield could be due to the loss of the required gramicidin analogue along with the impurities during purification procedures, because of the aggregation property of GA and its analogues. An effective purification step was by preparative TLC.

The carbon-13 magnetic resonance spectrum in dimethyl sulfoxide of the synthetic analogue is given in Figure 2 along with a spectrum for comparison of the naturally occurring gramicidin A in which the resonances have been assigned (Fossel et al., 1974; Prasad et al., 1982a). Note the decrease in intensity of the Val α-*C*H resonance at 57.5 ppm along with the corresponding increase in the Ala α-*C*H signal at 48.4 ppm. The substitution of two valine methyl carbons for one of alanine is more difficult to assign as both resonances appear at approximately equivalent chemical shifts (19–17 ppm). Also, note that no extraneous peaks are observed in the spectrum of the synthetic analogue and that all other signals are present in the correct position and proportion, which verifies the proper synthesis and purity of [L-Ala^7]gramicidin A. The proton magnetic resonance spectrum of the same sample is shown in Figure 3 again in comparison to a spectrum of the natural material with assignments (Glickson et al., 1972). The loss of Val β-C*H* intensity at 1.9 ppm and Val γ-CH_3 intensity at 0.8 ppm along with the increase in the Ala β-CH_3 signal at 1.2 ppm are clearly seen, which verifies the proper alanine for valine substitution. The α-C*H* resonances are less clearly seen as the Leu, Val, and Ala signals are overlapping (4.4–4.0 ppm); however, an increase in intensity is observed toward the upfield side of this envelope, which would also correspond to the loss of a valine and the addition

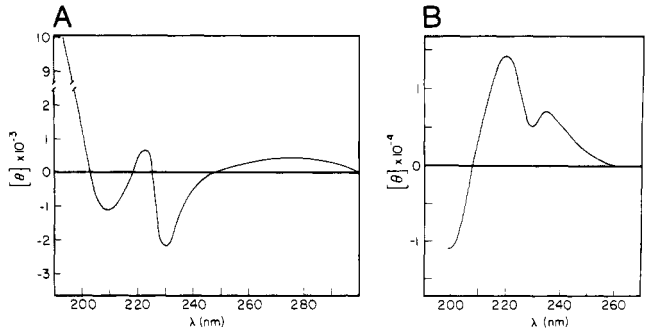

FIGURE 4: Circular dichroism spectra of (A) synthetic [L-Ala^7]GA in methanol and (B) a lysolecithin-packaged synthetic [L-Ala^7]GA showing the characteristic spectrum of the channel state for an essentially complete incorporation.

of an alanine residue. Interestingly, the 3:1 nonequivalence of the indole near 10.8 ppm is removed in the analogue, reflecting an altered side-chain distribution in this solvent. Again, all other resonances are present with no extraneous signals, confirming the synthesis and purity of the product.

The circular dichroism spectrum in methanol of the [L-Ala^7]gramicidin analogue is given in Figure 4A, where it may be seen that this peptide gives a spectrum very similar to that observed for both HPLC-purified natural gramicidin A and for synthetic gramicidin A (Prasad et al., 1982a). In figure 4B is shown the CD spectrum of the synthetic [L-Ala^7]GA packaged into lysolecithin bilayers. This curve is typical for samples in which the peptide has been incorporated into lipid in the proper channel conformation, which is necessary for ion interaction (Urry et al., 1979; Masotti et al., 1980). The concentration of channels in this preparation was found to be 2 mM, and the sodium-23 chemical shift and line width were 4.8 ppm and 195 Hz, respectively. A CD pattern indicating a completely incorporated state was never obtained with either [Phe^9]GA or [Phe^{11}]GA (GB) (Prasad et al., 1983). This observation supports the view with regard to channel formation in lipid bilayer suspensions that the GA molecule tolerates structural changes in the N-terminal half more readily than in the C-terminal part where the tryptophans are located (Prasad et al., 1983). Due to the successful incorporation of [L-Ala^7]GA into phospholipid bilayers, a determination of the binding and rate constants can be carried out with this analogue.

Since the study of the single-channel conductance data reports individual channel events, it becomes essential to have the peptides in a highly purified state. At the picomolar concentrations used in the investigation, a very low level of contamination could give rise to erroneous data. It is necessary to keep this in mind while dealing with single-channel properties. For that reason, after the analogue was purified to at least more than 95% homogeneity by TLC, ^{1}H NMR, and ^{13}C NMR standards, the main peak observed on HPLC was collected and repeatedly passed through an analytical HPLC column to ensure the greatest purity for BLM studies. The chromatogram along with the natural gramicidin mixture and a synthetic GA analogue is presented in Figure 5. When Val^7 is substituted by a less hydrophobic Ala, one can expect a reduced retention time on an ODS HPLC column. This is the result. [L-Ala^7]GA has a retention time of 6.75 min as compared to 8.67 min for synthetic GA.

The single-channel sweeps obtained with [L-Ala^7]GA displayed, strikingly, almost identical channel steps. This may be seen from a typical sweep shown in Figure 6B. Accordingly, the analysis of these sweeps resulted in the conductance histogram displayed in Figure 6A. The dominant peak at 28

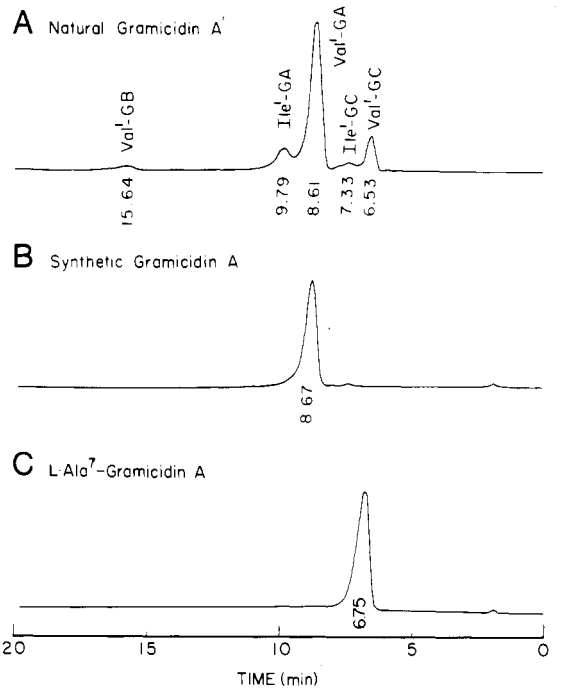

FIGURE 5: High-performance liquid chromatograms of (A) a natural gramicidin mixture, (B) a wholly synthetic GA, and (C) synthetic [L-Ala^7]GA on a Zorbax-ODS analytical column with a 15% H_2O/CH_3OH solvent system at room temperature.

pS is quite sharp with most of the events falling within the conductance range 26–30 pS. This can be contrasted with the conductance histogram obtained for synthetic GA at 26.8 °C under similar conditions (Figure 6A). While only 31% of the total events fall within a 1.75-pS window at the center of the peak for GA, 76% of the events occur within an identical window width for [L-Ala^7]GA. The automated analysis of the sweeps enables also the lifetime histograms to be derived. By taking only those single-channel events with conductance in the range of 27.1–28.85 pS and by using a bin size of 0.1 s, the lifetime histogram shown in Figure 6C is obtained. The inset in the figure shows a plot of ln of frequency against lifetime. An optimum least-squares fit through the initial 17 points (covering 70% of the events) yields a mean single-channel lifetime of 1.4 s for [L-Ala^7]GA, as compared to 0.7 s obtained for synthetic gramicidin A under similar conditions.

DISCUSSION

The approach of this Laboratory to the determination of the unique ionic mechanism of gramicidin channel transport is to use physical methods, independent of the single-channel current measurements themselves, to determine the location of ion binding sites within the channel and to determine the ion binding constants and rate constants relative to interaction with those ion binding sites. A key element in this approach is to be as sure as possible that exactly the same conducting state, or if necessary the same most narrow distribution of conducting states, is being characterized by all methods. This requires as a first step that a single analogue is being characterized. As a second step, an analogue is sought that has the most narrow distribution of single-channel currents. As a third step, the analogue must be able to be incorporated into a lipid system in which more than 90% of the molecules are in the channel state. These are a severely restricting set of criteria. Many analogues can form conducting channels in planar lipid bilayers even though their fraction of conducting to nonconducting state may differ by many orders of magnitude. While it is of little consequence in characterizing

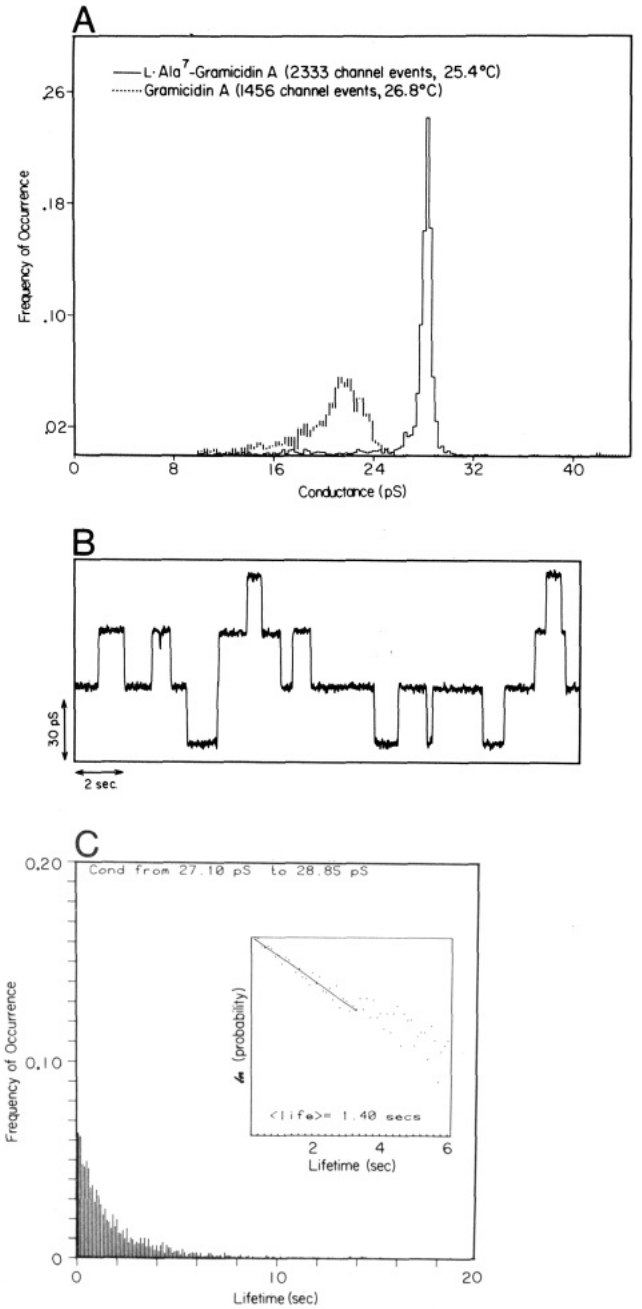

FIGURE 6: (A) Single-channel conductance histograms for the synthetic [L-Ala^7]GA (solid line) and the synthetic GA (dotted line). (B) Single-channel conductance trace of [L-Ala^7]GA in diphytanoyllecithin/*n*-decane membranes; about 76% of the channel events fall in the narrow 1.75-pS conductance window for [L-Ala^7]GA as compared to 31% of the events for the synthetic GA. (C) Lifetime histogram for [L-Ala^7]GA employing the single-channel conductance events in the range of 27.1–28.85 pS with a bin size of 0.1 s; the inset shows a plot of ln of frequency against channel lifetime and gives a mean lifetime of 1.4 s whereas half that value is obtained for gramicidin A.

single-channel conductance properties to have a very low and variable fraction of molecules in the channel state, it is important to have essentially all of the molecules in the channel state when physical methods are used to characterize bulk suspensions of channels in lipid. Also, the analogues as is the case for gramicidin A itself, can exhibit a wide range of single-channel conductance values. This has been referred to as a dispersity or multiplicity of conductance states. When binding processes and especially rate processes are characterized by NMR, however, there becomes the concern as to whether the values obtained are most relevant to the average channel conductance, to the most probable channel conductance, or to a particular lesser probable, much lower conducting state. Both of these problems appear to be most satisfactorily resolved by the [L-Ala^7]GA analogue. As shown in Figure 4B, it incorporates very well as the channel state into phospholipid. As shown in Figure 6A, it exhibits the most narrow distribution of single-channel currents of any analogue compared thus far under identical conditions.

In Figure 1 is shown a side-chain distribution for the gramicidin A channel structure, and the view is taken, in general, that the greater the number of energetically allowed side-chain distributions the greater the dispersity of single-channel currents. Changes in single-channel current magnitudes are considered to be most sensitive to tryptophan side-chain orientations. Of interest here is the possible orientations of the Trp^{13} side chain, which, as shown, is directed away from the channel as is the case for the Trp^{11} side chain (see lower part of Figure 1). Another orientation is exhibited by the Trp^9 and Trp^{15} side chains where the indole is directed toward the channel junction and the Trp^9 side chain overlays the small Ala^3 side chain. What are of primary concern are the $i \pm 6$ side-chain interactions. With the [L-Ala^7]GA analogue, the Trp^{13} side chain could also be oriented like the Trp^9 side chain and overlay the Ala^7 side chain. An additional orientation such as this, if of similar energy to the orientation in Figure 1, could lead to more allowed side-chain distributions. If the additional orientation were significantly energetically more favored, then a decrease in the number of side-chain distributions could occur and the dispersity of single-channel currents could be expected to decrease. As a decrease in dispersity and a significant increase in probability of a narrow conductance band occur, the latter interpretation would seem to be required.

As a bonus that is apparent in Figure 6, [L-Ala^7]GA exhibits the highest single-channel conductance yet observed for an analogue of GA. That it does so has immediate implications with regard to rate-limiting mechanisms. If an analogue under identical conditions can exhibit a higher conductance than gramicidin A, then effects such as a diffusion-controlled association step and interfacial polarization (Andersen, 1983a,b) cannot be rate-limiting for those conditions. At present, [L-Ala^7]GA appears to be the most favorable analogue with which to pursue a detailed determination of ionic mechanism.

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