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Ion Interactions in (1-^{13}C)D-Val^{8} and D-Leu^{14} Analogs of Gramicidin A, the Helix Sense of the Channel and Location of Ion Binding Sites

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Summary. Ion-induced chemical shifts in the carbonyl carbon resonances of synthesized and verified (1-^{13}C)D-Val^{8} gramicidin A and (1-^{13}C)D-Leu^{14} gramicidin A are utilized in combination with the previously determined location of the ion binding sites of the gramicidin A channel (using the carbonyls of L-residues) to determine that the helix sense of the gramicidin A channel is left-handed. Having resolved the handedness issue, the location of the ion binding sites (which are fundamental to understanding the mechanism of ion transport) are further delineated with the results indicating two sites separated by just over 20 Å. Furthermore, the demonstration that the divalent barium ion interacts at the binding site while not being transported through the channel is used to argue that the mechanism of monovalent *vs.* divalent cation selectivity is due to the positive image force contribution to the central barrier.

Key Words helix sense · gramicidin channel · binding sites · carbon-13 magnetic resonance · peptide synthesis · ion selectivity

Introduction

The primary structure of 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}-NHCH$_2$CH$_2$OH, was determined by Sarges and Witkop [21] and verified by Gross and Witkop [10]. The effect of gramicidin A (GA) in inducing ionic conductance across lipid bilayer membranes was first shown in the pioneering studies of Mueller and Rudin [16], and the interesting on-off step conductances exhibited by gramicidin A, which reflect the formation and disruption of a conducting transmembrane structure, were first observed by Hladky and Haydon [12].

Conformations of peptides comprised of alternating L- and D-amino acid residues have been independently proposed by Ramachandran and Chandrasekharan [18, 19] and by this Laboratory [23, 29]. Also in the original papers [23, 29] this Laboratory specifically proposed the dominant structure of the gramicidin A channel to be that of two left-handed $\pi^{6}_{L,D}$-helices which dimerized by head-to-head (formyl end to formyl end) hydrogen bonding. Due to the hydrogen bonding pattern between turns of the helices, the name was changed to β-helices [24, 25] and most technically the proposed channel structure was termed a single-stranded $\beta^{6.3}_{3,3}$-helix [30]. Subsequently Veatch and Blout and colleagues described parallel and antiparallel double stranded β-helices for Gramicidin A [9, 36, 37], and the central question became which was the correct channel structure, one of the head-to-head dimerized single stranded β-helices or one of the double stranded parallel or antiparallel β-helices.

While conformational energy arguments were presented as not being conclusive in deciding between the single stranded and double stranded β-helices of gramicidin A [7], the effects of chemical modification of gramicidin A on transport properties, including the original head-to-head malonyl dimer [4, 29], charged derivatives [1–3, 6], and the effects of the N-acetyl derivative [22, 23], were the basis for concluding that the end-to-end, and specifically the head-to-head, hydrogen bonded single stranded β-helices were the dominant channel-forming structure of gramicidin A in planar bilayers. It is now well accepted that this is the dominant structure for gramicidin A in the lipid bilayer membrane [2, 39], and even the crystal structure of gramicidin A complexed with K^+ and with Cs^+ [14], though yet at low resolution, yields a tubular structure with a length and radius which is within tenths of an Å of the previously proposed single-stranded $\beta^{6.3}_{3,3}$-helical channel [24, 25, 30].

While the details of the ionic mechanisms of transport are being determined such as the binding constants for a number of monovalent cations [8, 13, 20], the rate constants for sodium ions moving

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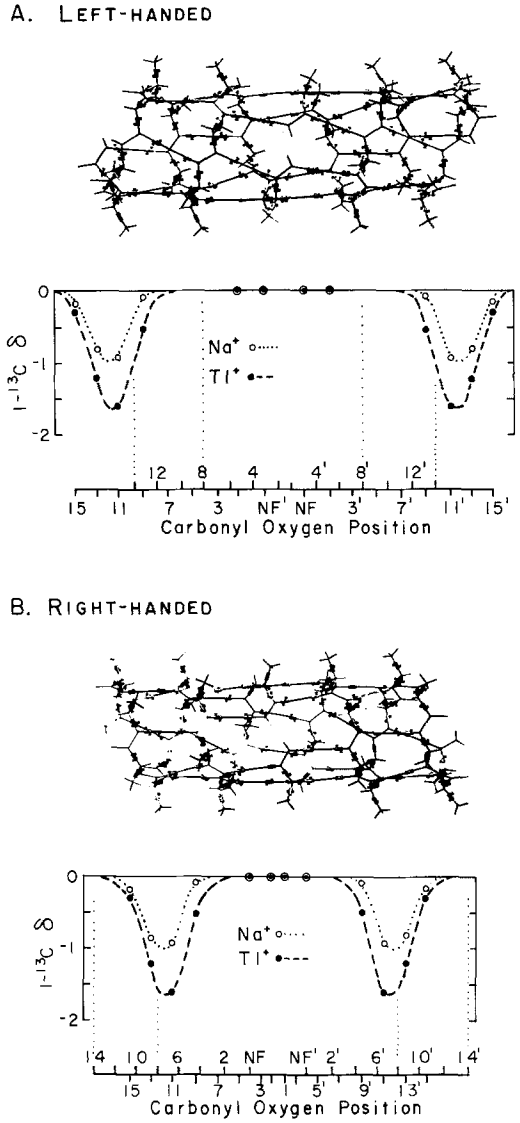

Fig. 1. Single stranded head to head dimerized $\beta^{6,3}_{3,3}$– helix of Gramcidin A. Wire models of (*A*) left- and (*B*) right-handed helix structures of a gramicidin A channel. The amino acid side chains are represented by methyl groups in order to display the peptide backbone more clearly. Also shown for each helix is a scale indicating the position of the L- and D-amino acid carbonyl oxygens in the channel along with the 1-^{13}C chemical shifts previously reported for the formyl, Val^1, Trp^9, Trp^{11}, Trp^{13}, and Trp^{15} carbonyl carbons in the presence of sodium and thallium ions [31]. Note that the positions for the D-Leu^{14} and D-Val^8 carbonyl oxygens are marked with dotted lines

in and out of the channel [34, 35], the location and number of binding sites within the channel [31] and the rates of ion movement within the channel [11], there is an important structural detail that has yet to be proven. This is the helix sense of the channel. Is it left-handed as originally proposed [23, 29] or is it right-handed? There is evidence favoring the left-handed structure. For example, the circular dichroism spectrum of gramicidin A incorporated as the channel state into phospholipid structures would be consistent with a left-handed structure [15, 32], yet this is not conclusive as the required detailed calculations have not been carried out.

Given the single-stranded β-helical structures, the handedness can be demonstrated by determining the combination of L- and D-amino acid residue carbonyls which occur at the ion binding site. This is demonstrated in Fig. 1. In Fig. 1 *A* is the left-handed channel structure and below it are the ion-induced chemical shifts of 1-^{13}C enriched L-amino acid residues of gramicidin A which localize the binding sites [31]. In Fig. 1 *B*, however, is the right-handed channel structure and the location of sites should this be the helix sense. The important thing to note is that the D-amino acid residues that have carbonyls at the binding site differ depending on the helix sense. If the structure is left-handed, the D-Leu^{14} carbonyl is near the binding site, whereas if the structure is right-handed, the D-Val^8 carbonyl is at the binding site. The present manuscript reports the ion-induced carbon-13 chemical shifts of two isotopes of the channel, (1-^{13}C) D-Leu^{14} gramicidin A and (1-^{13}C)D-Val^8 gramicidin A. The results confirm the helix sense of the channel and the general channel structure, allow determination of the distance between sites, add evidence to the absence of binding sites between the two located sites, and provide additional information on the basis of monovalent *vs.* divalent ion selectivity.

Abbreviations

TFA – trifluoroacetic acid
DCC – dicyclohexylcarbodiimide
Et_3N – triethylamine
DMF – dimethylformamide
P_2O_5 – phosphorus pentoxide
C/M/A – chloroform/methanol/glacial acetic acid
HPLC – high performance liquid chromotography
tlc – thin layer chromotography
TloAc – thallium acetate

Materials and Methods

Syntheses

Boc-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}-*(1-*^{13}*C)*D-*Leu*^{14}-L-*Trp*^{15}-*NHCH*$_2$*CH*$_2$*OH (I) and Boc*-L-*Val*^{1}-*Gly*^{2}-L-*Ala*^{3}-D-*Leu*^{4}-L-*Ala*^{5}-D-*Val*^{6}-L-*Val*^{7}-*(1-*^{13}*C)*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*$_2$*CH*$_2$*OH (II)*. The synthesis of analog I was carried out on a Vega-Model 50 Solid Phase Peptide Synthesizer using Boc-Trp-Resin (2.5 g). The resin used contained a total of 1.45 mmol of Boc-Trp-OH (0.58 mmol/g) esterified to a 1% cross-linked resin (Peninsula Laboratories, Inc., San Carlos, Calif.). The synthesis of analog II was also carried out on the Vega-Model 50 using Boc-Trp-Resin (2.5 g) containing a total of 1.475 mmol of Boc-Trp-OH

(0.59 mmol/g) esterified to a 1% cross-linked resin. The following steps used previously for the synthesis of isotopically labeled gramicidin A [17] were performed to incorporate each amino acid: (1) washing with CH_2Cl_2 (3 × 3 min), 40 ml; (2) 33% TFA, 6% anisole, 5% 1,2 ethanedithiol in CH_2Cl_2 (3 × 13 min), 35 ml; (3) washing with CH_2Cl_2 (6 × 3 min), 40 ml; (4) 5% di-isopropylethylamine in CH_2Cl_2 (3 × 6 min) 40 ml; (5) washing with CH_2Cl_2 (6 × 3 min), 40 ml; (6) 2.5-fold excess Boc-amino acid, 2.5-fold excess DCC in CH_2Cl_2 and reacted for 6 hr; (7) washing with CH_2Cl_2 (3 × 3 min), 40 ml; (8) washing with EtOH (3 × 3 min), 40 ml; (9) washing with CH_2Cl_2 (6 × 3 min), 40 ml; (10) 1.5-fold excess of Boc-amino acid, 1.5-fold excess of DCC in CH_2Cl_2 and reacted for 3 hr; (11) washing with CH_2Cl_2 (3 × 3 min), 40 ml; (12) washing with EtOH (3 × 3 min), 40 ml; (13) check by Kaiser test, to estimate completeness of reaction; (14) washing with CH_2Cl_2 (3 × 3 min) 40 ml; (15) acetylation with 10 eq. acetic anhydride and 5 eq. Et_3N in CH_2Cl_2 (60 min) to terminate any unreacted chains; (16) washing with CH_2Cl_2 (6 × 3 min), 40 ml, and continue at step 2 for the next Boc-AA coupling. It was necessary to first dissolve Boc-D-Leu-OH and Boc-Trp-OH in a minimum volume of DMF and then dilute with CH_2Cl_2 for coupling.

The Boc-Val[1]...Trp[15]-resin (4.39 g for analog I and 4.23 g for analog II) was suspended in a mixture of methanol (100 ml) plus distilled ethanolamine (50 ml) in a sealed flask and stirred at 60 °C for 40 hr. The resin was filtered, washed with methanol and dried. Methanol was removed from the filtrate; the peptide was precipitated by the addition of water; the precipitate was filtered, washed with H_2O, and dried (1.23 g for analog I and 1.42 g for analog II). Further treatment of the resin with ethanolamine under the same conditions proved to remove the remaining peptide from the resin.

Desformyl-(1-^{13}C)D-Leu^{14} Gramicidin A (III) and Desformyl-(1-^{13}C)D-Val^{8} Gramicidin A (IV). In a solution of 33% TFA, 5% 1,2 ethanedithiol, and 6% anisole in CH_2Cl_2 (50 ml), the Boc-gramicidin was stirred for 1 hr under nitrogen atmosphere. The solvent was removed under reduced pressure and the peptide dried over P_2O_5 and NaOH in a vacuum desiccator. The peptide in TFA salt form was taken in methanol (25 ml) and passed through a column of AG 50 W-X2 (H^+ form) ion-exchange resin (50 ml) which had previously been equilibrated with methanol. Ion exchange chromotography was done at 6 °C. The unreacted gramicidin was eluted with methanol (500 ml) and elution was continued with 2.0 N methanolic-ammonia (1600 ml MeOH + 320 ml concentrated ammonium hydroxide) to obtain 680 mg of III and 1.04 g of IV.

Formyl-(1-^{13}C)D-Leu^{14} Gramicidin A (V) and Formyl-(1-^{13}C)D-Val^{8}-Gramicidin A (VI). The desformyl-gramicidin A was dissolved in 95–97% formic acid (9.3 ml for III and 14.3 for IV) and cooled to 0° with ice-water. While stirring, acetic anhydride (2.7 ml for III and 4.2 ml for IV) was added by drops over a period of 20 min. Stirring continued for 30 min at 0° and 4 hr at room temperature. The solvent was removed and the residue dried over P_2O_5 and NaOH. Dried peptide was taken in methanol (9 ml for V and 14 ml for VI) and treated with 1 N NaOH (1.8 ml for V and 2.7 ml for VI) for 1 hr. The reaction mixture was passed through a column of AG 50W-X2 (H^+ form) ion-exchange resin (50 ml) and eluted with methanol to obtain 362 mg of formulated (1-^{13}C)D-Leu^{14}-gramicidin A and 401 mg of formylated (1-^{13}C)D-Val^{8}-gramicidin A. The formyl peptide was purified by preparative tlc using a solvent system of C/M/A (85:15:3) for plate developing. The tlc band with an R_f value corresponding to that of natural gramicidin A was separated and the peptide extracted with 15% MeOH in acetone. The solvent was removed, the residue taken in a small volume of methanol, and the peptide precipitated by the addition of water. The precipitate was filtered, washed with water and dried to obtain 140 mg of formyl-(1-^{13}C)D-Leu^{14}-gramicidin A and 68 mg of formyl-(1-^{13}C)D-Val^{8} gramicidin A. For removal of silica gel, the formyl-gramicidin A was taken in 3 ml of methanol and loaded on a column of LH-20 Sephadex. A flow rate of 22.5 ml/hr and fractions of 5.0 ml gave 95 mg of pure formyl-(1-^{13}C)D-leu^{14}-gramicidin A and 34 mg of pure formyl-(1-^{13}C)D-Val^{8}-gramicidin A.

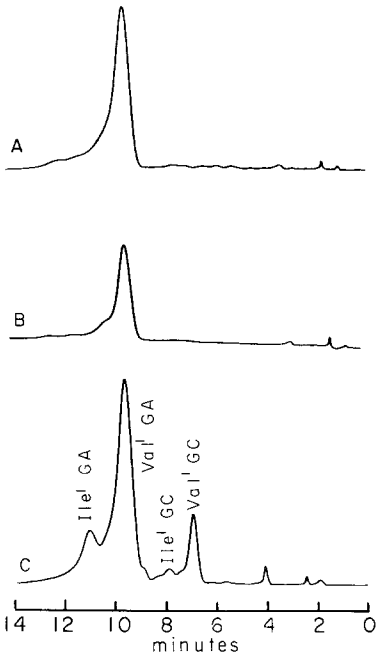

Fig. 2. HPLC of (*A*) synthetic (1-^{13}C)D-Leu^{14} gramicidin A; (*B*) synthetic (1-^{13}C)D-Val^{8}-gramicidin A; and (*C*) the natural gramicidin A′ mixture showing the A, C and Ile analogs. The chromatographs were performed on a DuPont Zorbax ODS analytical column using 10% water in methanol solvent system at room temperature

Characterization

In Fig. 2 HPLC data of the two synthetic formyl gramicidin analogs are compared with that of natural gramicidin (ICN Pharmaceuticals, Inc.). The solvent system, 10% H_2O in MeOH gave the optimum resolution. A DuPont Zorbax ODS analytical column (4.6 mm × 25 cm), at room temperature was used. The retention time for the synthetic analogs paralleled exactly that of the Val[1]-gramicidin fraction in the natural material. TLC gave a single spot in C/M/A (85:15:3) with an R_f value the same as that of natural GA.

To further assess the purity of the synthetic gramicidin, the carbon-13 magnetic resonance spectra at 30 °C in dimethyl-d_6 sulphoxide (Merck, Sharp & Dohme, Montreal, Canada) was observed. The spectra are presented in Fig. 3 along with a corresponding spectrum for the natural gramicidin A′, and were obtained on a JEOL PFT-100 pulse-Fourier transform spectrometer operating at 25 MHz with an internal deuterium lock and complete proton noise decoupling.

Phospholipid Packaging

The (1-^{13}C)D-Val^{8} GA (V) and the (1-^{13}C)D-Leu^{14} GA (VI) were separately incorporated into L-α-lysolecithin (Avanti Bio-

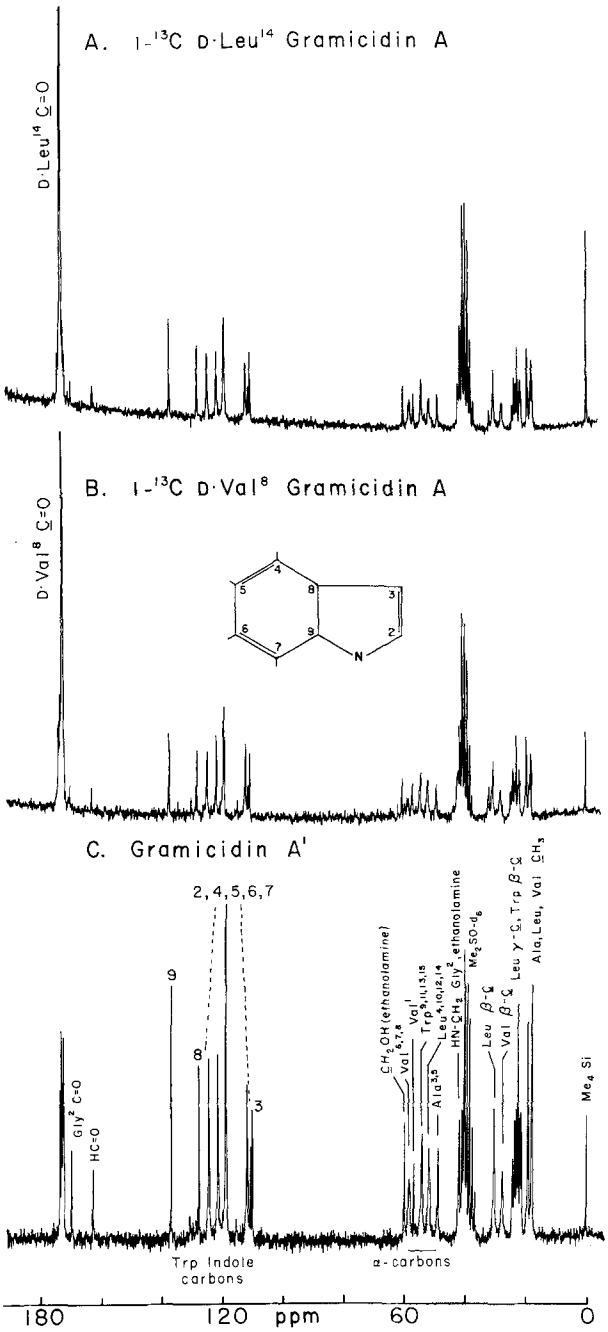

Fig. 3. Carbon-13 nuclear magnetic resonance spectra at 25 MHz of (*A*) (1-^{13}C)D-Leu^{14} gramicidin A; (*B*) (1-^{13}C)D-Val^{8} gramicidin A; and (*C*) the natural gramicidin A′ mixture in dimethyl-d_6-sulphoxide at 30 °C. Assignments are shown for the spectrum of the natural gramicidin [9]. The intense signal in the low field region of the two synthetic gramicidins corresponds to the enriched 1-^{13}C carbonyl carbon of each analog

chemicals, Birmingham, Ala.) structures as described previously [32, 33]. Each sample was heated for at least 20 hr at 70 °C with 0.5 mM NaCl present. After heat incorporation, the correct gramicidin channel forming state [15, 32, 33, 38] was confirmed by circular dichroism as seen in Fig. 4. Also, the sodium-23 chemical shift at 0.5 mM NaCl was determined for these samples and was found to be indicative of interaction with the channel

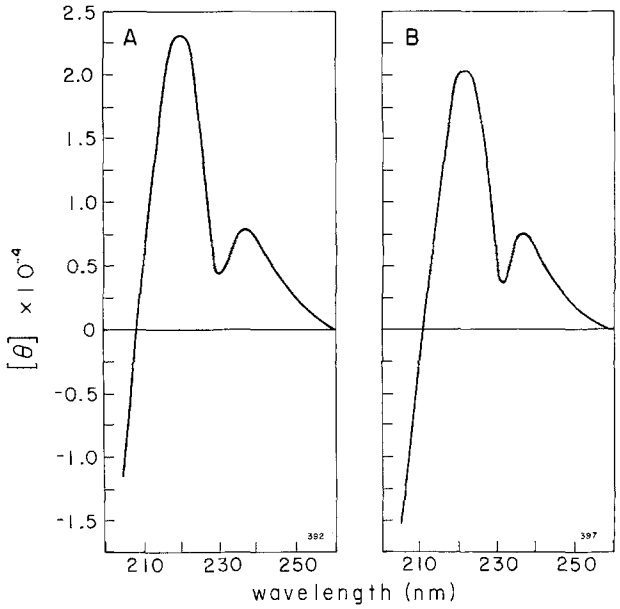

Fig. 4. Circular dichroism spectra of phospholipid packaged: (*A*) (1-^{13}C)D-Leu^{14} gramicidin A, and (*B*) (1-^{13}C)D-Val^{8} gramicidin A, with both showing the characteristic spectrum of the channel state

state. The concentration of the gramicidin associated with the phospholipid was determined by dispersion of an aliquot of each sample in methanol and observing the ultraviolet spectra, using an ε_{282} of 22,500 liter/mol-cm as the standard. The channel concentrations determined were 3.43 mM for (1-^{13}C)D-Val^{8} GA and 3.37 mM for (1-^{13}C)D-Leu^{14} GA.

Carbon-13 Nuclear Magnetic Resonance

The carbon-13 nuclear magnetic resonance spectra of the phospholipid incorporated gramicidins were obtained on a JEOL FX-100 pulse-Fourier transform spectrometer also operating at 25 MHz with internal deuterium lock and complete proton noise decoupling. The spectra were accumulated at 70 °C in order to increase the mobility of the sample so as to facilitate observation of the ^{13}C enriched backbone carbonyl. However, especially in the case of the (1-^{13}C)D-Val^{8} GA, the signals were very broad and weak; therefore, no exponential window function was applied to the free induction decay before the Fourier transform and each observation pulse given was 90° with repetition times allowing for complete relaxation before the next pulse. These accumulation parameters favorably affected both the signal width and the signal-to-noise ratio of the backbone carbonyl.

The procedure for the ion titration of the channels was followed as previously reported [31]. The carbon-13 enriched carbonyl chemical shift having no ion interaction is taken as the spectra in the presence of 0.5 mM NaCl. For single sodium ion occupancy, the NaCl concentration was raised to 100 mM; for thallium ion, 83 mM TlOAc was added to the samples, and for barium ion 0.2, 0.4, 0.6, 0.8 and 1.0 M concentrations were obtained by additions of barium chloride.

Results and Discussion

As demonstrated in Materials and Methods, the syntheses have been verified by thin-layer chroma-

tography, by high performance liquid chromatography (*see* Fig. 2) and by carbon-13 nuclear magnetic resonance spectra in dimethyl sulfoxide (*see* Fig. 3), and the incorporation of the channel state into lysolecithin structures was verified by the circular dichroism spectra (*see* Fig. 4) and the sodium-23 magnetic resonance chemical shift at 0.5 mM NaCl. Therefore for both carbon-13 isotopes of gramicidin A, the channel state was clearly obtained.

The results of the carbon-13 magnetic resonance studies on the ion-induced carbonyl carbon chemical shifts are shown in Fig. 5. It is apparent in Fig. 5 *B* that neither 99 mM NaCl, 83 mM T10Ac, nor 1.0 M $BaCl_2$ causes any shifting or dramatic broadening of the Val^8 carbonyl carbon resonance. In Fig. 5 *A* the Leu^{14} carbonyl carbon resonance shows a detectable shift with 99 mM NaCl, a substantial shift due to T10Ac, and a large shift with broadening due to 1.0 M $BaCl_2$. The downfield shift moving under the lipid carbonyl resonance was seen on titrating by 0.2 M steps with $BaCl_2$. Interestingly, the circular dichroism spectra are essentially unchanged by any of the ion additions demonstrating that the channel state is retained in all cases in the presence of ions. Thus it can be concluded that the Val^8 carbonyl is not located at an ion binding site, whereas the Leu^{14} carbonyl is clearly at a cation interaction site.

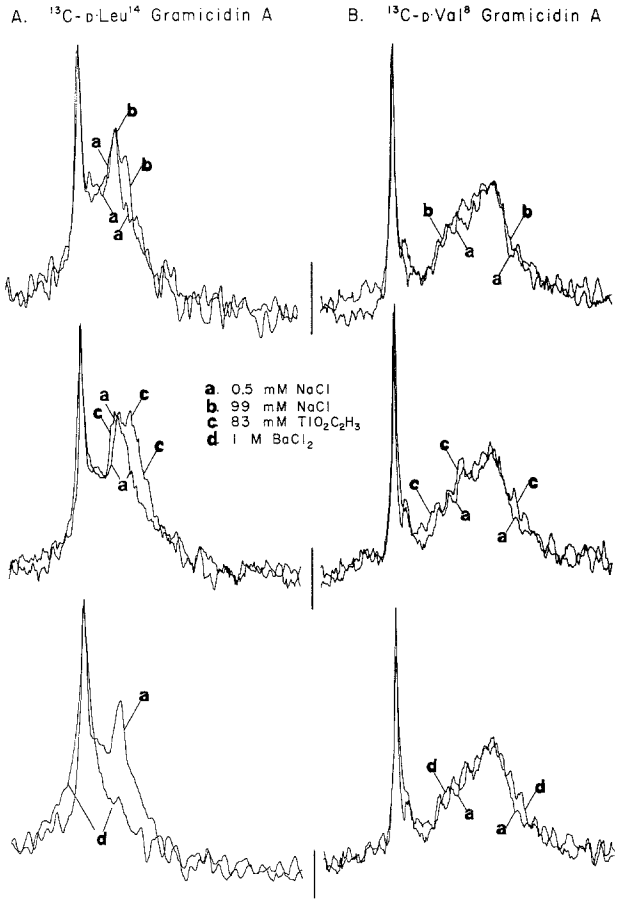

Fig. 5. Carbon-13 nuclear magnetic resonance spectra at 25 MHz of the carbonyl carbon region (177–165 ppm) of phospholipid packaged: (*A*) (1-^{13}C)D-Leu^{14} gramicidin A, and (*B*) (1-^{13}C)D-Val^8 gramicidin A channels. Chemical shifts are given with respect to external hexamethyldisiloxane. The broad high field resonances correspond to the 1-^{13}C enriched peptide backbone carbonyls, and the low field peak is due to the lysolecithin carbonyl carbon. Shown for each is the effect of the addition of sodium, thallium, and barium ion upon the chemical shift of the enriched position. The spectra observed in the presence of 100 mM NaCl (*b*) are uppermost with those observed in the presence of 83 mM $TlO_2C_2H_3$ (*c*) and 1 M $BaCl_2$ (*d*) shown below, respectively. Each spectrum is shown overlayed with a reference spectrum in which the sodium ion concentration is 0.5 mM (*a*) and where the change in chemical shift of the peptide carbonyl is taken as zero. Note that the lipid carbonyl does not exhibit an appreciable change in chemical shift for any case. All spectra were accumulated at 70 °C

Helix Sense of the Gramicidin A Channel

As previously demonstrated with six different isotopically labeled gramicidin A preparations, namely ^{13}C-formyl, (1-^{13}C)L-Val^1, (1-^{13}C)L-Trp^9, (1-^{13}C)L-Trp^{11}, (1-^{13}C)L-Trp^{13} and (1-^{13}C)L-Trp^{15} [31], and as shown in Fig. 1, the cation binding sites are symmetrically related and are seen to be centered between the Trp^{11} and Trp^{13} carbonyls but most proximal to the Trp^{11} carbonyl. With the helix sense of the channel being right-handed as shown in Fig. 1 *B*, the Val^8 carbonyl is found between the Trp^{11} and Trp^{13} carbonyls and, as such, would be optimally placed for an ion-induced chemical shift of its carbonyl carbon resonance. Concommitantly, the Leu^{14} carbonyl would be outside of the binding site region and would not be expected to exhibit ion-induced shifts of its resonance. The absence of any shift in the carbonyl carbon resonance of the Val^8 residue, argues against the right-handed helix as a possible structure. If the helix were left-handed as shown in Fig. 1 *A*, the Val^8 carbonyl would be well removed from a binding site and the Leu^{14} carbonyl would be found between the Trp^9 and Trp^{11} carbonyls, not optimally placed but within the range where an ion-induced shift might be observed. Indeed clear shifts in the Leu^{14} carbonyl carbon resonance are observed for thallium ion and for barium ion. Thus the ion-induced chemical shift data with thallium and barium ions, both the absence of any observed shift in the Val^8 carbonyl carbon resonance and the observation of a shift in the Leu^{14} carbonyl carbon resonance, demonstrate the helix

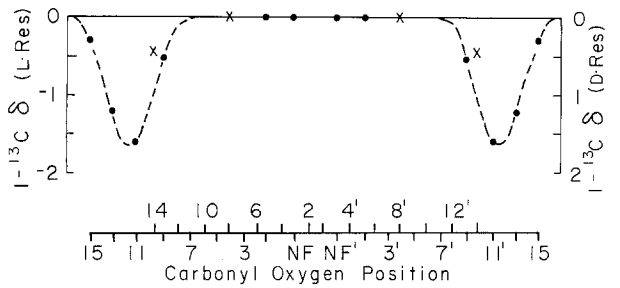

Fig. 6. Thallium ion-induced chemical shifts (●) of the 1-^{13}C L-amino acid residues as previously reported [31] for the left-handed helical structure of the gramicidin A channel. The positions along the helix axis of the carbonyl oxygens are indicated. Thallium ion induced chemical shifts for the (1-^{13}C)D-Leu^{14} and the (1-^{13}C)D-Val^{8} carbonyl carbons are shown with an x on the plot. The left ordinate is for the L-residue chemical shifts and the right ordinate indicates the shift for the D-residues

sense of the gramicidin A channel to be left-handed.

The general aspects of the backbone structure of the gramicidin A channel have now been completely determined by experimental results and have been shown to be as originally proposed [23, 29], i.e., the head-to-head (amino end to amino end) dimerization of two monomers [1–4, 6, 22, 29, 39] each in the left-handed $\beta^{6,3}_{3,3}$ helical conformation (this manuscript). The original considerations which led to the proposal of the left-handed helix over the right-handed helix [23, 29] were the apparent lesser crowding of the eight L-amino acid side chains in the left-handed helix as compared to the six D-amino acid side chains and the facile dimerization of the left-handed helices. Calculations are currently in progress (C.M. Venkatachalam and D.W. Urry, *work in progress*) using the complete side chains in order to attempt quantitation of these factors and any others that may become apparent.

Location of the Ion Binding Sites

With the demonstration of the left-handed helix sense and with a 1.5 to 1.6 Å translation along the helix axis for each L-D dipeptide pair [7, 25], the distance between ion binding sites is just slightly more than 20Å. A value of 21Å for the separation of ion binding sites has been reported for crystals of gramicidin A complexed with K^+ and with Cs^+ [14]. As shown in Fig. 6, the addition of the zero ion induced chemical shift for the Val^8 carbonyl carbon resonance, when added to the previous data for the formyl and Val^1 carbonyls [31], further limits the possibilities for intervening binding sites for the ions and conditions considered here. This data, locating the ion binding sites, is crucial to understanding the mechanism of ion transport by gramicidin A and at this stage clearly demonstrates the existence of two sites.

Monovalent vs. Divalent Ion Selectivity

While divalent cations are not transported by the gramicidin A channel, there is transport data which indicates that calcium ions and barium ions do interact to decrease the single-channel currents [5]. The interaction of barium ion with the Leu^{14} carbonyl, demonstrated in this report, as well as the interaction observed with the 1-^{13}C Trp analogues of gramicidin A (work in progress) indicate that the divalent ion can enter the ion binding site. Since the interaction of divalent ions with the binding site of the lipid incorporated channel state is intact, it is necessary to look for other reasons as to why divalent ions are not transported. It has already been proposed that the lipid dielectric constant, which contributes most markedly to the central barrier but much less at the binding sites, is responsible for the lack of divalent ion conductance [26, 27]. The free energy height of the central barrier for sodium ion is between 8 and 9 kcal/mole [28, 34]. If we assume that half of this barrier is due to the low dielectric constant of the lipid membrane and utilize the charge squared dependence for the free energy of solvation then the central barrier could be expected to be greater than 20 kcal/mole for divalent ions and the divalent ion conductance would be less by a factor of 10^{-8} or more. Thus it is quite easy to understand the exclusion, for example, of calcium ion while sodium ion with the same radius is transported.

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