Binding of Alkaline Cations to the Double-Helical Form of Gramicidin

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ABSTRACT Gramicidin is a polypeptide antibiotic that forms monovalent cation-specific channels in membrane environments. In organic solvents and in lipids containing unsaturated fatty acid chains, it forms a double-helical "pore" structure, in which two monomers are intertwined. This form of gramicidin can bind two cations inside its lumen, and the crystal structures of both an ion complex and an ion-free form have been determined. In this study, we have used circular dichroism (CD) spectroscopy to examine the binding mechanism and the binding constants (K_1 and K_2) of cations to gramicidin in the double helical form in methanol solution. The dramatic change in optical rotation in the far-ultraviolet CD spectrum of gramicidin provides a useful tool for monitoring the binding. The binding mechanism appears to involve a large conformation change associated with the binding of ions to the first of the two sites. The calculated values for the K_1 binding constants for alkaline cations are considerably smaller than the K_2 binding constants. The order of binding affinity for alkaline cations is similar to that for the helical dimer "channel" form of gramicidin, i.e., $Cs^+ \approx Rb^+ \gg K^+ > Li^+$, but in comparison to the helical dimer form, the binding to double-helical dimers is dominated by a cation size-dependent conformational change in the gramicidin structure.

INTRODUCTION

Gramicidin is a linear hydrophobic polypeptide that is synthesized by *Bacillus brevis* (Hotchkiss and Dubos, 1940). Gramicidin has three main naturally occurring isoforms, designated gramicidin A, B, and C. The primary sequences of gramicidin (Sarges and Witkop, 1965) are

formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-

Val-L-Trp-D-Leu-L-xxx-D-Leu-L-Trp-D-Leu-L-

Trp-ethanolamine,

where xxx is Trp for gramicidin A, Phe for gramicidin B, and Tyr for gramicidin C, and the naturally occurring mixture, designated gramicidin D, consists of about 80% gramicidin A, 5% gramicidin B, and 15% gramicidin C (Weinstein et al., 1980). The alternating L- and D-amino acids in the sequence produce an unusual β-helical secondary structure in which all of the side chains are located on the periphery of a cylindrical-type structure.

The physiological function of gramicidin remains unclear (Harold and Baarda, 1967; Paulus et al., 1979; Bohg and Ristow, 1986), but, in membrane environments, gramicidin has been found to form ion channels that are specific for the transfer of monovalent cations (Myers and Haydon, 1972; Andersen, 1984). In lipids composed primarily of saturated fatty acids, gramicidin is a right-handed twisted $\beta^{6.3}$-helical dimer (called the "channel" form) with two monomers connected N-terminus to N-terminus by six intermolecular hydrogen bonds (Arseniev et al., 1985a; Urry, 1971; Roux and Karplus, 1994). On the other hand, in lipids composed of unsaturated fatty acids and in organic solvents, gramicidin mainly adopts double-helical structures (called the "pore" form) in which the two monomers are interwound along their entire length and connected by a total of 28 intermolecular hydrogen bonds (Wallace, 1990; Sychev et al., 1993). The double-helical form of gramicidin forms stable complexes with monovalent cations (Wallace, 1990; Wallace and Ravikumar, 1988). From ^{13}C nuclear magnetic resonance (NMR) and x-ray crystallography studies (Urry, 1984; Wallace and Ravikumar, 1988) it has been recognized that the carbonyl groups of tryptophan residues at position 11 are the principal sites for the binding of cations in both the helical dimer and double-helix forms.

The binding mechanism of the channel form has been extensively investigated by using NMR-detected competitive binding of Tl-205 (Hinton et al., 1986), Li-7 (Urry et al., 1983), Na-23 (Cornelis and Laszlo, 1979; Venkatachalam and Urry, 1980; Urry et al., 1982), K-39 (Urry, 1984), and Cs-133 (Urry, 1984) conductance measurements (Eisenman et al., 1978; Eisenman and Horn, 1983; Dani and Levitt, 1981; Levitt et al., 1978; Russell et al., 1986) and dialysis methods (Levitt et al., 1978; Veatch and Durkin, 1980). The values obtained by the different approaches, for example for Cs^+, have not been very consistent and vary over the range from 700 M^{-1}, measured by conductance (Eisenman and Horn, 1983), to 54 M^{-1}, measured by Tl-205 NMR (Hinton et al., 1986). Although the measured binding constants are variable, the relative binding affinities found for different cations are generally consistent, with the series being $Cs^+ \approx Rb^+ \gg K^+ > Na^+ > Li^+$. In general, the cations bind more strongly to the first binding site than to the second one. No significant conformational change accompanied the binding as detected by circular dichroism (CD) spectroscopy (Wallace et al., 1981). For the gramici-

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din double-helical form, the binding affinity and mechanism have not yet been investigated, despite the fact that the crystal structures for both ion-free and ion-bound forms of the double helix have been known for a number of years (Langs, 1988; Wallace and Ravikumar, 1988).

The crystal structures of ion-free gramicidin (Langs, 1988) and the cesium complex of gramicidin (Wallace and Ravikumar, 1988) are very different, although both are left-handed antiparallel double helices. The complex, with 6.4 residues/turn, is much wider in diameter and shorter in length than the ion-free form, with 5.6 residues/turn, which has an irregular lumen that is too narrow to accommodate alkaline cations in certain regions (Smart et al., 1993). The differences in ψ and ϕ angles between the two forms produce quite different far-ultraviolet CD spectra, and the ellipticity changes from negative in the ion-free form to positive in the ion-bound form. Such a dramatic change in the CD spectrum provides a means for studying the ion-binding mechanism. Furthermore, both cesium- (Wallace and Ravikumar, 1988) and potassium-gramicidin (Doyle and Wallace, 1994; Doyle and Wallace, manuscript in preparation) complexes of the molecule have been examined by x-ray crystallography, and these two ion complexes show subtle but significant structural differences.

In this study it was found that the binding of monovalent cations to double-helical gramicidin in methanol involves two probably equivalent interacting cation sites, either of which produces a conformational change upon ion binding, and that large cations have much tighter binding constants than small cations. The change in conformation plays an important role in determining the binding affinity for the different alkaline cations. The binding affinity for different alkaline cations is roughly related to the size of cations in the series from Cs^+ to Li^+, similar to that observed for the helical dimer form.

MATERIALS AND METHODS

Materials

Gramicidin D was purchased from ICN Biochemicals. Spectrograde methanol and methyl-d_3-alcohol were purchased from Aldrich Chemical Company (Gillingham, England). Cesium chloride was purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). Potassium chloride and sodium chloride were purchased from BDH Chemicals (Poole, England). Rubidium chloride and lithium chloride were purchased from Sigma Chemical Company (St. Louis, MO). All chemicals were reagent grade and were used without further purification.

Solution preparation

Methanolic stock solutions of 15 mg/ml gramicidin were prepared. The stock solutions of salt were made by dissolving them in methanol at the highest concentrations possible because of the different solubilities of the alkaline cations. The different ratios of gramicidin/salt solutions were prepared by adding the appropriate proportions of alkaline cation stock solutions and methanol to the gramicidin stock solution to produce a final gramicidin concentration of 1.5 mg/ml. The final concentration of gramicidin was reduced to 0.75 mg/ml in the Li^+, Na^+, and K^+ experiments to achieve saturation of binding. This is because the binding constants of these ions are much lower than that for Cs^+ and Rb^+, and because the solubility of these ions in methanol is much reduced. Thus, the lower gramicidin concentrations are necessary to achieve saturation of binding. The final concentration of gramicidin in each solution was determined by using uv-visible absorption spectroscopy with an absorption coefficient of 22,000 M^{-1} cm^{-1} at 280 nm in the same cell used for the CD measurements.

For NMR spectroscopy, CsCl and gramicidin solutions were prepared by dissolving 1.7 mg of CsCl in 1 ml methyl-d_3-alcohol and sonicating until it was completely dissolved. Then an amount of gramicidin was added to the solution to produce a final concentration of 10 mM gramicidin. The final molar ratio of gramicidin to Cs was 1:1.

Circular dichroism spectroscopy

Circular dichroism spectra were recorded using an AVIV 62DS spectropolarimeter with a large-angle detector geometry. The optical rotation was calibrated using *d*-10-camphorsulfonic acid at wavelengths 192.5 and 290 nm. The wavelength was calibrated with benzene vapor. All measurements were made in Suprasil quartz cells with pathlengths of either 0.01 or 0.05 cm.

In general, data were collected in the wavelength range from 200 to 250 nm at 0.5-nm increments. Every CD spectrum reported is the average obtained from at least three individual samples and three repeated measurements of each sample. The reported circular dichroism spectra were corrected for baseline using a solvent containing the same concentration of salt, and then smoothed using a Savitsky-Golay filter. All measurements were carried out at 25.0 ± 0.2°C .

Nuclear magnetic resonance spectroscopy

The one-dimensional ^1H NMR spectra were obtained using a JEOL XS-500 mode pulse 500 MHz spectrometer at 20.0 ± 0.2°C. Spectra are reported using tetramethylsilane at 0 ppm or methanol at 3.3 ppm as references.

Binding polynomial

The mechanism for binding alkaline cations to gramicidin was assumed to be a two-binding-site process in which each monovalent cation binds to a carbonyl group in one end of the double helix and subsequently inevitably alters the conformation, as suggested from the crystal structure of the gramicidin-cesium complex (Wallace and Ravikumar, 1988). Because only antiparallel double-helical forms have been reported in ion complex studies (Wallace and Ravikumar, 1988; Arseniev et al., 1985), and our NMR result (see below) also shows only a single species, the bound form is assumed to be a single species with an antiparallel double-helical conformation. The general equations for the cation binding are as follows:

$$GA_0 + L \leftrightarrows GA_iL \qquad K_1 = \frac{[GA_iL]}{[GA_0]\cdot[L]} \tag{1}$$

$$GA_iL + L \leftrightarrows GA_iL_2 \qquad K_2 = \frac{[GA_iL_2]}{[GA_iL]\cdot[L]} \tag{2}$$

Where GA_o is the ion-free gramicidin, including all dimeric conformers and any monomers; GA_i is the ion-bound dimer in which the conformation is different from any of the conformations found for the ion-free form of GA_0; and L represents the monovalent cations. From the NMR results (see later), it is clear that under the conditions used, the concentration of monomers is very small, so GA_o is essentially equivalent to the concentration of ion-free dimers. The different dimeric conformers in GA_o can be interconverted to each other. The CD spectrum at any concentration is the net combination of the ion-free (GA_o) and ion-bound (GA_i) forms weighted by their concentrations. Two general models based on linked two-site

binding, and consistent with the x-ray structural data, are proposed as possible binding mechanisms.

1. The first model is one in which the two binding sites (equivalent for the first ion binding) interact so that the second ion binds with a different binding constant than the first, although it does not matter which site of the two sites was initially occupied. This would be expected for symmetrical sites with equivalent accessibility and structures, but where the first ion binding affected the second ion binding (by direct electrostatic effects, indirectly through effects on nearby solvent molecules, or in this case, by causing a conformational change in the molecule). The degree of saturation, Y, is then described as the fraction of gramicidin dimers in the GA_i state, which includes both singly and doubly occupied dimers as follows:

$$Y = \frac{K_1[L] + 2K_1K_2[L]^2}{1 + K_1[L] + K_1K_2[L]^2} \quad (3)$$

where [L] is the concentration of the monovalent cations, and K_1 and K_2 are the equilibrium binding constants for the first and second binding sites, respectively.

2. The second model assumes that the two binding sites of different affinities (due to different site structure or accessibility, for instance) are independent of each other. In this case, the degree of saturation can be written as

$$Y = \frac{K_1[L]}{1 + K_1[L]} + \frac{K_2[L]}{1 + K_2[L]} \quad (4)$$

where the values of K_1 and K_2 would be equal if the two binding sites were equivalent and noninteracting.

To distinguish between these two models, the degree of saturation as a function of ion concentration was determined experimentally. It was calculated from the optical rotation change between the ion-free gramicidin and ion-bound complexes at a wavelength of 228 nm as follows:

$$Y(x) = \frac{\theta_o - \theta_x}{\theta_o - \theta_\infty}, \quad (5)$$

where θ_∞ is the ellipticity at a fully saturating ion concentration, θ_0 is the ellipticity in the absence of ions, and θ_x is the ellipticity for each of the ion concentrations, respectively, and is assumed here to vary linearly with $[GA_i]$.

Nonlinear curve fitting

The binding parameters were calculated using the nonlinear curve fitting function in the Origin program (MicroCal Software, Northampton, MA, USA). This nonlinear fitting program uses the Levenberg-Marquardt (LM) nonlinear least-squares fitting algorithm. In the initial fitting stage, the Simplex method, which was set at a 100-cycle run, was used to calculate the initial input parameters to set up the rough parameter region. Then these parameters were used to set the constraint conditions for the curve fitting. A 0.95 confidence level target was set to constrain the quality of the curve fitting. The final fitting parameters were obtained when the value of χ^2 was less than 0.05 and the parameters and the errors for the parameters reached a convergent and steady state.

RESULTS

NMR spectroscopy

Fig. 1 shows the NMR spectra obtained for gramicidin in methanol with and without CsCl present. As was found by Arseniev and his co-workers (1985b) when they titrated gramicidin in chloroform/methanol with cesium, the NMR spectra with and without cations are significantly different. First, the four discrete N-H protons of the indole rings of tryptophan residues located at 10–11 ppm in the gramicidin-Cs sample are not observed in the ion-free gramicidin spectrum, where they are shifted upfield and are closely coincident. Second, the region around 8–8.2 ppm, where there are the multiple peaks in the ion-free gramicidin solution, becomes a single intense peak in the complexed form. This signal is assigned as the formyl group and is an indicator of the change in gramicidin conformation from multiple species to a single species, because ion-free gramicidin has been found to exist primarily as four interconvertible dimeric con-

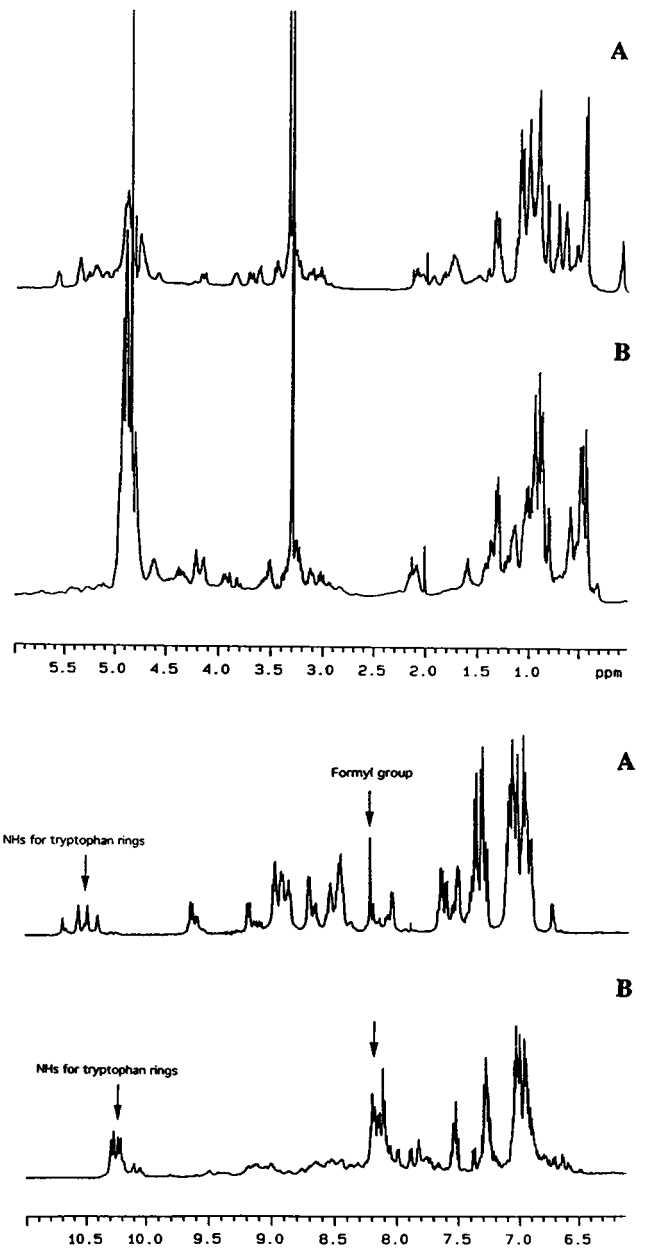

FIGURE 1 Five hundred megahertz ^{1}H-NMR spectra for cesium-bound (*A*) and cesium-free (*B*) gramicidin. The concentration of gramicidin is 10 mM in d_3-methanol.

formers in methanol solution (Veatch et al., 1974; Veatch and Blout, 1974). The different dimeric conformations of gramicidin have been assigned as left-handed antiparallel (named species 3), left-handed parallel (species 1 and 2, only different in helical stagger), and right-handed parallel double helices (Veatch and Blout, 1974). Although there is an equilibrium between the ion-free dimeric species and monomer species (Veatch, 1974), at the concentrations used in these experiments the proportion of monomers as detected in the NMR spectra, which would have been obvious in the peaks from the formyl and indole N-H protons, was very small. This is similar to the situation found by Fossel et al. (1974). From the noise levels in the spectra, we can estimate the monomer: dimer ratio to be considerably less than 0.05, and thus the amount of monomer present in these experiments, and by extrapolation, in the CD experiments, is essentially inconsequential.

CD spectroscopy

Three titrations in the far-ultraviolet region of the CD spectra of gramicidin with CsCl, Cs_2CO_3, and LiCl are shown in Fig. 2, *A*, *B*, and *C*, respectively. Fig. 3 shows the fully ion-saturated titration CD spectra for different alkaline cations. For the ion-free forms, there are two negative peaks in the spectrum, whereas in the fully saturated forms there is a single positive peak (Fig. 3). Similar titrations were obtained for all the alkaline cations examined, except sodium. In that case, the CD spectrum could not be converted into the fully saturated form, because of the low solubility of the NaCl in methanol, which prevented preparation of a sufficiently concentrated salt solution. The two negative bands located about 213 and 229 nm are assigned as the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions. The positive peak is generally located at about 228 nm in the ion-saturated form and shifted to slightly longer wavelengths with increasing concentrations of alkaline ions. The spectral change reaches a maximum (fully saturated) when the salt concentrations are about 16 mM, 8 mM, and 2.8 M for CsCl, Cs_2CO_3, and LiCl, respectively. Further additions of ions had no effect on the spectra. Similar types of spectral changes were observed in all of the ion titrations. The maximum changes in ellipticity for the potassium and lithium cations are much smaller than those for cesium and rubidium cations. This suggests that the conformational change associated with binding large cations may be significantly greater than that which occurs when small monovalent cations are bound. The molar ratios at which the gramicidin/monovalent cation complexes become saturated are 20 for CsCl and Cs_2CO_3 and 7000 for LiCl. Therefore, the dramatic change in the ellipticity in this far-ultraviolet region of the CD spectra provides a means of investigating the binding constants of monovalent cations to gramicidin.

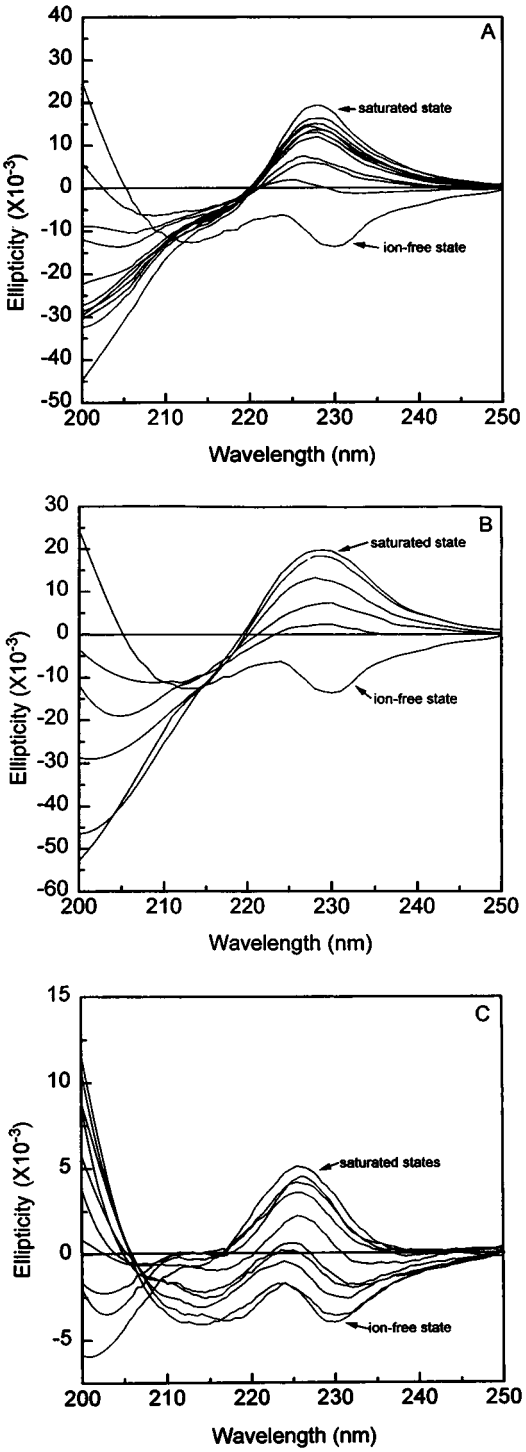

FIGURE 2 The far-ultraviolet circular dichroism spectra of gramicidin, showing the titrations with (*A*) cesium chloride, (*B*) cesium carbonate, and (*C*) lithium chloride. In *A*, the gramicidin concentration was 0.8 mM and cesium chloride concentrations (mM) were 0 (*lowermost spectrum*), 0.8, 4.4, 5.6, 8.4, 9, 9.6, 11.2, 12.5, 14, and 16 (*uppermost spectrum*). In *B*, the gramicidin concentration was 0.8 mM and cesium carbonate concentrations (mM) were 0 (*lowermost spectrum*), 0.8, 1.6, 3.2, 6.4, and 8 (*uppermost spectrum*). In C, the gramicidin concentration was 0.4 mM and lithium chloride concentrations (M) were 0 (*lowermost spectrum*), 0.2, 0.5, 0.75, 0.8, 1.3, 1.6, 1.9, 2.4, and 2.8 (*uppermost spectrum*). With the increasing concentration of ions, the spectra convert from two negative peaks in the ion-free form to a single positive peak in ion-saturated complexes.

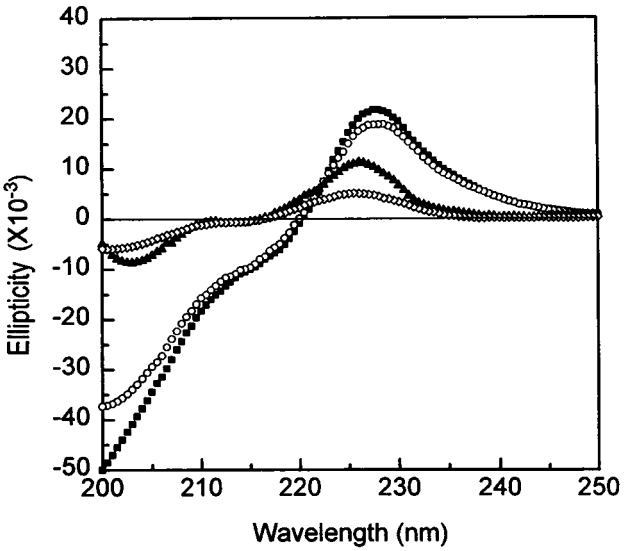

FIGURE 3 Far-ultraviolet circular dichroism spectra for saturated cesium- (■), rubidium- (○), potassium- (▲), and lithium- (◇) gramicidin complexes. The saturated molar ratios for each complex are 20:1 for Cs^+/gramicidin, 30:1 for Rb^+/gramicidin, 2000:1 for K^+/gramicidin, and 7000:1 for Li^+/gramicidin.

Titration curve fitting

Fig. 4, *a* and *b*, shows the binding curves (the degree of saturation versus concentration) for CsCl and LiCl, respectively. In both figures, the solid lines represent the best-fit curves from interacting two-site binding (model A) and the dotted lines represent the best-fit curves from independent two-site binding (model B). It can be seen that the interacting two-binding-site model is a better fit than the independent two-site model. In general, the curves for the independent two binding-site model significantly underestimate the values for high concentrations of cations and overestimate the values for low concentrations of cations in relation to the experimental data. For the interacting two binding-site model, all of the correlation coefficients are >0.98, with all points located within the 0.95 confidence range. The calculated curves for all experimental binding curves of different alkaline cations show an excellent fit in the low concentration range, but slightly underestimate the values at high concentrations. The associated binding constants, K_1 and K_2, estimated from the interacting two-binding-site model, and their corresponding correlation coefficients and χ^2 values, for all the cations are summarized in Table 1.

The binding constants calculated from the interacting two-binding-site model show that the first binding constant is considerably smaller than the second binding constant. The binding affinities are significantly different between the large cations such as cesium and rubidium, and the smaller alkaline cations. The binding constant for the most tightly binding cesium cation is about 70 times larger than the tightest lithium binding constant. The trend of the binding affinity decreasing with the size of cations is in accordance with the relative binding affinities previously seen for the helical dimer form of gramicidin. It should be noted that the binding constants calculated for both Cs salts are essentially the same (Table 1, and for Cs_2CO_3, K_1 = 360 M^{-1} and K_2 = 694 M^{-1}), thus demonstrating that this is a cation, not an anion, effect.

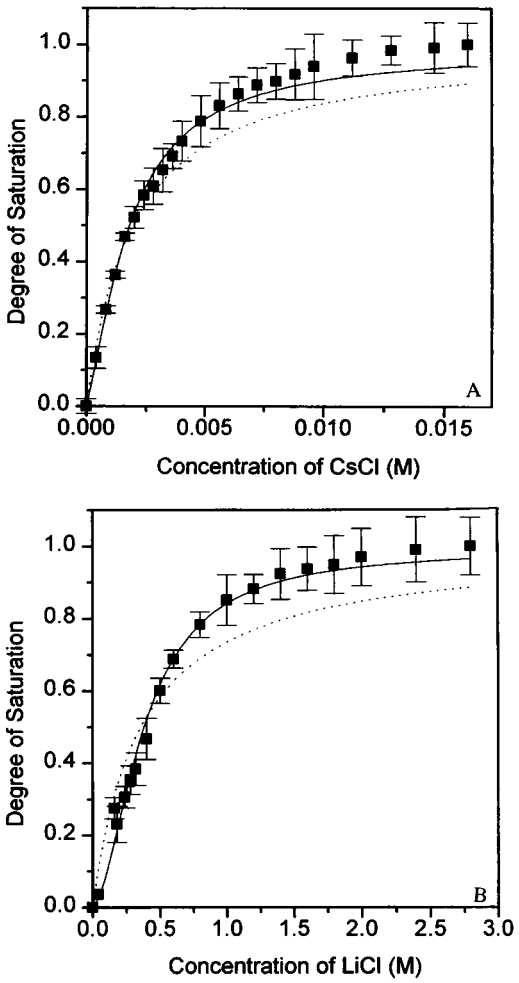

FIGURE 4 Nonlinear least-squares fits for the cesium chloride/gramicidin (*A*) and lithium chloride/gramicidin (*B*) titration curves. In each plot, the solid and dotted lines represent the fits using the two interacting binding site and two independent binding site models, respectively.

TABLE 1 Summary of first (K_1) and second (K_2) binding constants, correlation coefficients (R), and χ^2 values for different cation chloride/gramicidin complexes

	Cs^+	Rb^+	K^+	Li^+
K_1^*	394.2 (±29.4)	382.1 (±72.2)	11.9 (±3.9)	0.7 (±0.2)
K_2^*	744.6 (±51.3)	717.2 (±89.2)	22.74 (±6.13)	10.0 (±2.8)
R	0.991	0.980	0.982	0.997
χ^2	0.00183	0.0035	0.00204	0.00135

*Dimension is M^{-1}.

DISCUSSION

The aims of this study were to determine cation binding constants and to examine the possible mechanism of monovalent cation binding to the gramicidin double-helical form in methanol, in order to further our understanding of the ion interactions of this polypeptide. The binding of alkaline cations was of interest for two reasons: 1) The crystal structures of the Cs- and K-gramicidin double-helical ("pore") complexes produced from methanol solution have been well characterized (Wallace and Ravikumar, 1988; Doyle and Wallace, 1994). In addition, the crystal structure of the ion-free double-helical form of gramicidin has been determined (Langs, 1988). Hence, the different crystal structures provide a good reference for interpretation of the binding behavior, and 2) The phenomenon of alkaline cation binding to the helical dimer ("channel") form of gramicidin has been intensively investigated. The comparison between the two different forms of gramicidin interacting with the same types of ions can aid in our understanding of the transport and binding processes.

The crystal structures of gramicidin cannot provide a complete picture of how monovalent cations bind to gramicidin, however, because the two binding sites in the symmetric left-handed antiparallel double-helical structure appear to be indistinguishable and are fully occupied at the concentration of cations used to prepare the crystals. Therefore, to investigate the nature of the binding, we needed a method that could allow us to examine the dynamics of the system and compare the possible interacting and independent models for two binding sites.

The effect arises from cation, not anion binding

Besides cations, the possible effect of chloride anions must be considered, because the crystal structure of the Cs^+ gramicidin complex shows three Cl^- anions located at the center and two ends of the pore. Hence, the role of anions was examined by titration with salts containing the same cations but different anions. Similar results have been observed (see Fig. 2, *A* and *B*) for the binding of both cesium chloride and cesium carbonate (the carbonate anions of which are very unlikely to enter the gramicidin pore, because of their large size), as well as for cesium thiocyanate (a complex in which only one anion is found in the crystal structure; Doyle and Wallace, 1994), and for potassium hydroxide (the hydroxide anions of which cannot enter the pore). All have binding constants that are very similar to the results for the chloride salts. These results are consistent with the crystal structure, in which the binding sites for anions show no significant geometric distortion from an ideal helix, whereas the oxygen atoms of the nearby carbonyl groups show an obvious movement away from the helical axis and a direct binding to the cations, which suggests a much weaker interaction with the anions present.

The mechanism of binding alkaline cations to gramicidin

The change in the NMR signals of CsCl/gramicidin indicates that the conformation of gramicidin converts to a single species in the ion-bound form. It is apparent from NMR studies (Arseniev et al., 1985a; this work, data not shown) that reequilibration between the remaining ion-free species after each addition occurs within the time course of this experiment. The very different CD spectra of the ion-free and ion-bound forms shown in Fig. 2 are consistent with these observations. The CD spectrum of the ion-free form is of a mixture of four interconverting dimeric species in methanol (and possibly a small amount of monomer that is too small to be detected in the NMR spectrum). One of the dimeric forms is the stable structure found in the ion-free crystals (Veatch et al., 1974; Fossel et al., 1974; Langs, 1988). The CD spectrum of the ion-bound form shows the presence of a single double-helical species and is consistent with the monovalent cation-gramicidin complex in solution being the antiparallel double helix seen in the Cs-containing crystals (Wallace and Ravikumar, 1988). The x-ray diffraction studies (Wallace and Ravikumar, 1988; Langs, 1988) reveal that in crystals both the ion-free and Cs-gramicidin complex conformations are left-handed, antiparallel double helices, with the cesium complex having a larger helical pitch, which produces a shorter cylinder (26 Å) than the ion free form (35 Å).

In our calculation of binding constants, the possible effects arising from the existence of monomers are considered insignificant. Veatch and Blout (1974) proposed a rather qualitatively determined dimerization constant for gramicidin of $2 \times 10^2\ M^{-1}$. We find no evidence for monomeric species in our NMR spectra, especially in the formyl and indole NH regions, and thus would estimate the dimerization constant in methanol to be significantly larger than that previously reported. As previously discussed, the ion-complexed solution contains a single dimeric species. Therefore, the contribution of monomer-cation complexes can also be excluded.

The results in Fig. 4 clearly show that the interacting two-binding-site model best fits the behavior of the alkaline cation binding to the double-helical form of gramicidin. The binding affinity for the first cation was found to be much weaker than for the second one, because the first cation has to overcome the unfavorable geometry of the narrow ion-free form by producing the conformational change. The binding of the second cation is more favorable because of the absence of an effective structural barrier. This is consistent with the crystal structures (Fig. 5), in which the ion-bound form is much wider and shorter than the ion-free form. Accordingly, the interactive mechanism for the binding of alkaline cations to gramicidin can be generally interpreted as three ion states with two distinct conformational states: conformation 1 (ion-free), conformation 2 with one ion bound, and conformation 2 with two ions bound, as shown in Fig. 5.

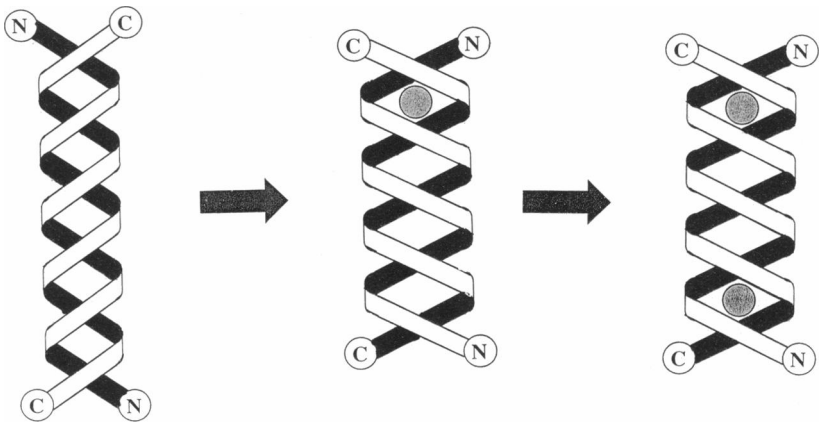

FIGURE 5 Schematic diagram showing the proposed two interacting binding site mechanism for monovalent cation binding to gramicidin. The binding is a three-state process, but has only two distinct gramicidin conformations between the ion-free and ion-bound forms.

The binding affinity is related to the size of the alkaline cations, because the binding constant is decreased in going from Cs^+ to Li^+. The difference in the binding constants between large and small alkaline cations is significant. This is consistent with the results shown in Fig. 3. The very different intensity of the CD spectra for the different saturated alkaline cation/gramicidin complexes suggests that the gramicidin internal radius is induced to be wider with the large cations, i.e. Cs^+ and Rb^+, than with the smaller cations, because the smaller size cations do not produce as large a positive CD signal at 228 nm as do the larger cations. The phenomena in Fig. 3 are also consistent with the previous suggestion (Kimball and Wallace, 1982) that the helical pitches are different for the different complexes, based on the expected difference in ellipticity for helices in general, as a function of number of residues per turn (Woody and Tinoco, 1967).

The binding constants of the Cs^+ ion for the double-helical form of gramicidin in a solution of methanol/chloroform have been estimated previously using ^1H NMR (Arseniev et al., 1985b). The reported binding constants were $K_1 = 10^4\ M^{-1}$ and $K_2 = 3 \times 10^3\ M^{-1}$ (no method of calculation cited). The larger K_1 constant suggests that the binding mechanism is not an interactive process. Their values are significantly different from our data (perhaps in part because of the different solvent system used) and inconsistent with the crystal structure and our NMR titration data in methanol solution (results not shown).

Comparison between ion binding of the double-helical and helical dimeric forms of gramicidin

According to several studies (Hinton et al., 1986; Urry et al., 1982, 1983; Cornelis and Laszlo, 1979; Venkatachalam and Urry, 1980; Urry, 1984), the helical dimer form of gramicidin has a binding behavior opposite to that of the gramicidin double-helical form. Its first binding constant is much larger than its second one. Furthermore, the binding affinity of the double-helical form for both Cs^+ and Rb^+ is much stronger than for the helical dimer form. On the other hand, the binding affinity of small cations to the double-helical form is much weaker than the same cations to the helical dimer form.

The difference between these two types of interactions by the two forms of gramicidin must be related to their structures. The helical dimer form does not undergo a conformational change upon ion binding (Wallace et al., 1981), so it must be possible for ions to bind to a preexisting site. In the double-helical form, the lumen is not sufficiently large to accommodate an ion, and therefore, an appropriate site must first be created. Once the conformational change occurs to permit the first ion to bind, it makes the binding at the second site easier. Because the helical dimer form effectively transfers the monovalent cations from the side of a membrane with higher ion concentrations to the side with lower concentrations of ions, the binding affinity for the second monovalent cation can be weaker than that of the first alkaline cation. In the helical dimer, the second site already exists but has a lower affinity because of electrostatic repulsion produced by the ion in the first site. Analyses of the internal radii of these different types of gramicidin (Smart et al., 1993), based on the structures from x-ray diffraction and NMR studies, show that the ion-free form has the narrowest lumen structure, whereas the ion-bound double-helical form and the channel form have similar dimensions, which can accommodate ions. The rather loose $\beta^{6.3}$-helical structure of the gramicidin helical dimer means there should be no major differences in the binding affinities of different-sized cations, which is consistent with the rather similar values of K_1 and K_2 measured by NMR for this form (Hinton et al., 1986). Finally, the double-helical form of gramicidin would appear to be better suited for ion binding than ion transfer, and this may account for the lower single-channel conductance reported for the double-helical form (Ovchinnikov and Ivanov, 1983).

CONCLUSIONS

In addition to providing an estimate of the equilibrium binding constants, these studies have also provided insight into the nature of the ion-induced conformation change in

the molecule. It has been found that there exists only one major species in the form of gramicidin-monovalent cation complexes. The binding of alkaline cations to the gramicidin double-helical dimers produces a change in conformation. This change plays a key role in the ion-binding mechanism. The binding of the first cation induces the widening of the double-helical dimer. The second cation then binds to the widened double-helical dimer. Consequently, the first binding constant is far weaker than the second binding constant. The relative binding affinities of alkaline cations are generally similar to the helical dimeric form of gramicidin, i.e., following the series $Cs^+ \approx Rb^+ \gg K^+ > Li^+$.

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