Shifting the Equilibrium Mixture of Gramicidin Double Helices Toward a Single Conformation with Multivalent Cationic Salts

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ABSTRACT The conformation of the polypeptide antibiotic gramicidin is greatly influenced by its environment. In methanol, it exists as an equilibrium mixture of four interwound double-helical conformers that differ in their handedness, chain orientation, and alignment. Upon the addition of multivalent cationic salts, there is a shift in the equilibrium to a single conformer, which was monitored in this study by circular dichroism spectroscopy. With increasing concentrations of multivalent cations, both the magnitude of the entire spectrum and the ratio of the 229-nm to the 210-nm peak were increased. The spectral change is not related to the charge on the cation, but appears to be related to the cationic radius, with the maximum change in ellipticity occurring for cations with a radius of ~1 Å. The effect requires the presence of an anion whose radius is greater than that of a fluoride ion, but is otherwise not a function of anion type. It is postulated that multivalent cations interact with a binding site in one of the conformers, known as species 1 (a left-handed, parallel, no stagger double helix), stabilizing a modified form of this type of structure.

INTRODUCTION

The hydrophobic polypeptide gramicidin, whose primary sequence is (Sarges and Witkop, 1965)

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

forms helical dimeric channels in lipid bilayers. The alternating L and D configurations of the amino acids place all of the hydrophobic side chains on the surface of the dimer, thus creating an ion passage through the middle of the structure, with the lumen formed by the carbonyl and amide groups of the polypeptide backbone. Gramicidin channels specifically conduct monovalent cations, but divalent cations block monovalent cation conduction through the channel.

In organic solvents, the lack of order imposed by the solvent, as compared to the anisotropic environment of the lipid bilayer, allows gramicidin to adopt four different types of double-helical conformations that are in equilibrium with each other. Each of these consists of two interwound monomers ~31 Å in length, with 5.6 residues per turn, held together by 28 hydrogen bonds arranged in a β-sheet-like pattern (Veatch et al., 1974; Bystrov and Arseniev, 1988; Langs, 1988; Langs et al., 1991). The conformers differ from one another in the handedness of their helices, the relative orientation of the monomers within the dimer (parallel or antiparallel), and the overlap stagger between the monomers. These different conformers have been shown to have distinct circular dichroism (CD) spectra (Veatch et al., 1974). The net CD spectrum of the equilibrium mixture in solution (Wallace, 1983) is the linear summation of the spectra of the different components, weighted by their relative ratios present at equilibrium. Upon binding monovalent cations in methanol, the conformers undergo a major conformational change, which has been monitored by CD spectroscopy (Chen and Wallace, 1996). Crystalline complexes with cesium (Wallace and Ravikumar, 1988) and potassium (Doyle and Wallace, 1997) have shown that gramicidin/monovalent cation complexes are also double helices, but in this case have 6.4 residues per turn, and are thus shorter and fatter structures than those found in the absence of ions.

In the present study we have followed binding of multivalent cationic salts to gramicidin in methanol solution by circular dichroism spectroscopy. There is a shift in the equilibrium mixture of the four conformers found in ion-free solution to a single conformation in the mutivalent cation solutions. As a result of this work and an NMR study (Chen et al., 1996) on a calcium complex of gramicidin in methanol, we propose that multivalent cations interact specifically with a binding site on the parallel, in-register, left-handed, double-helical conformer (known as species 1), thus stabilizing a modified form of this structure. The effect of binding multivalent cations is thus very different from that seen upon binding monovalent cations in methanol (Chen and Wallace, 1996), where binding produces a single antiparallel double-helical conformation with an altered helical pitch, unlike any of the conformations found in ion-free solutions.

MATERIALS AND METHODS

All solutions were prepared with spectrograde methanol (Aldrich Chemical Company, Gillingham, England). Gramicidin D, a mixture of gramicidins

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A, B, and C in an approximate ratio of 80:5:15 (Weinstein et al., 1980), was obtained from ICN Biochemicals, and reagent grade salts were purchased from either Sigma Chemicals (St. Louis, MO) or Aldrich Chemical Company.

Gramicidin was dissolved in methanol at a concentration of 10 mg/ml. Concentrated stock solutions (10 mM) of each of the salts were prepared and mixed with the gramicidin stock solution (and methanol, as needed), in each case producing a solution with a final gramicidin concentration of 1 mg/ml.

An Aviv 62DS spectropolarimeter was used to record the spectra. Three scans over the wavelength range from 250 nm to 190 nm at intervals of 0.2 nm were obtained for each sample, and this procedure was repeated three times with independently made stock solutions. All measurements were made in Suprasil quartz cells with a path length of 0.01 cm. All nine spectra for each sample were averaged. From each averaged spectrum, the spectrum of the averaged baseline (methanol alone or methanol with the same concentration of salt) was subtracted, followed by smoothing with a Savitsky-Golay (1964) filter. All measurements are expressed in terms of ellipticity, because all experiments were carried out with the same concentration of polypeptide.

RESULTS

The spectra of gramicidin obtained in the presence of increasing concentrations of $SrCl_2$ are shown in Fig. 1 *A*. The intensities of both the negative peaks located near 229 nm and 210 nm and the positive peak near 195 nm increase with increasing $SrCl_2$ concentrations. Although all three peaks change, the ratio of the 229 nm peak to the 210 nm peak increases with increasing ionic strength. The maximum change in ellipticity occurs when ~4 mM $SrCl_2$ (or more) has been added. The final spectrum (Fig. 1 *A*, *lowermost curve*) strongly resembles that of the isolated ion-free species 1 reported by Veatch et al. (1974).

The changes in ellipticity at three different wavelengths, 195 nm, 210 nm, and 229 nm, were calculated as the difference in ellipticity between the salt-containing gramicidin solutions and the ion-free sample and plotted versus salt concentration (Fig. 1 *B*). For the peak near 229 nm, which shifts in wavelength slightly upon the addition of salt, the peak maximum was used in each case.

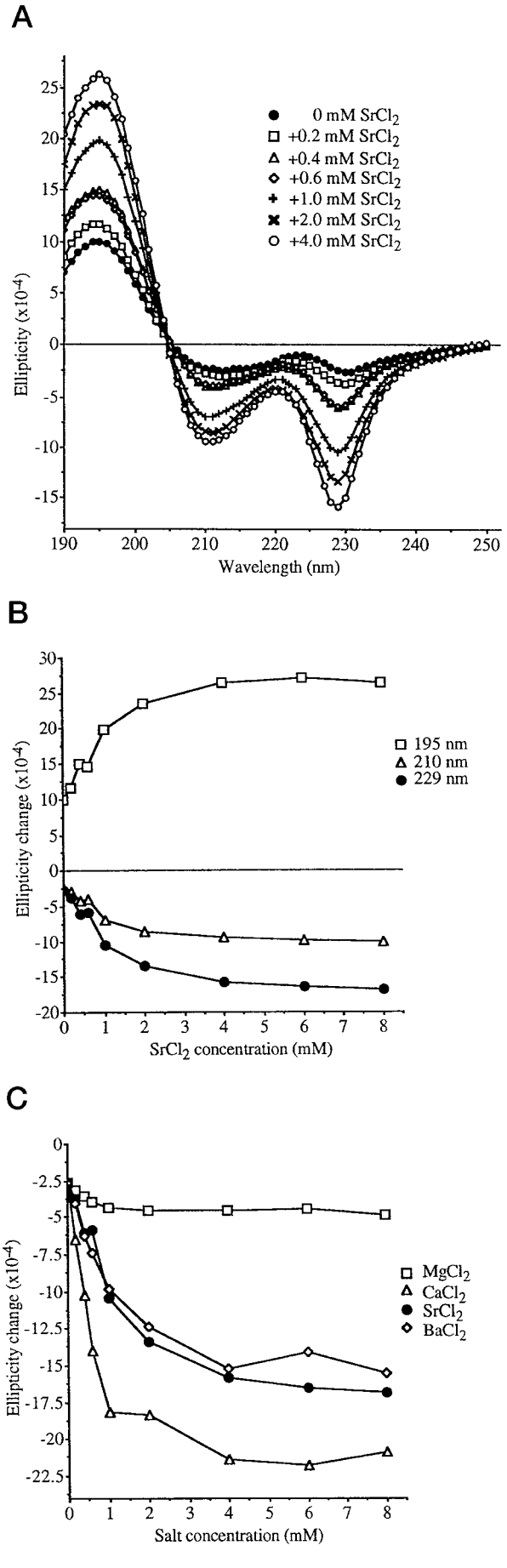
The same protocol was used to investigate the addition of the following group II chloride salts to determine whether divalent cations of different sizes (with the same anion: $MgCl_2$, $CaCl_2$, and $BaCl_2$) have different effects on the gramicidin conversion process (Fig. 1 *C*). The change in ellipticity at 229 nm was chosen to follow the transition, as measurements of the other peaks are noisier as a result of optical absorption by the salt solutions. $MgCl_2$ had very little effect on the spectrum of gramicidin, whereas $CaCl_2$ produced the greatest change in ellipticity of all the group II chloride salts. $BaCl_2$ and $SrCl_2$ were almost equally as effective at transforming the spectrum. These results rule out the +2 ionic charge as the sole cause of the spectral change, as all of these salts would be expected to produce the same change if this were the case. However, the size of the cation may have an important role to play, because the smallest cation examined (Mg^{2+}, Pauling ionic radius r = 0.66 Å) is a relatively poor converter, whereas the next cation in the group II series (Ca^{2+}, r = 0.99 Å) produces the greatest change.

The possibility that the anion could play a significant role in the gramicidin spectral change was examined by using the same cation with different halide anions (Fig. 2 *A*). Significantly, it can be seen that there is no effect on the spectrum of gramicidin in the presence of SrF_2, but the increase in magnitude is as effective for SrI_2 as it is for $SrCl_2$. No $SrBr_2$ was commercially available, so a similar titration was carried out with $BaBr_2$, which would allow a comparison with the $BaCl_2$ measurements (Fig. 1 *C*). The $BaBr_2$ results (Fig. 2 *B*) closely match those for the $BaCl_2$, and as the decrease in ellipticity due to the $BaCl_2$ salt is similar to that produced by the $SrCl_2$ salt (Fig. 1 *C*), the gramicidin conversion due to $SrBr_2$ is expected to match the results of the $SrCl_2$ and SrI_2 salts. It appears, then, that the alteration in the spectrum of gramicidin is an all-or-nothing response for the halide anions, with the change occurring for ions whose radii are larger than that of a fluoride ion (r = 1.33 Å). Indeed, a halide anion is not necessary for this process to take place, as a thiocyanate salt also produces the same transformation as the equivalent cationic halide salt (Fig. 2 *C*).

An examination of the effect of the cationic charge on the spectral change was also undertaken by titration with trivalent ($SmCl_3$) and tetravalent ($ZrCl_4$) cation chloride salts (Fig. 3 *A*). The magnitude of the spectral change at a particular ionic concentration does not appear to be proportional to the charge on the cation. In fact, the trivalent $SmCl_3$ salt is more effective at producing the spectral change than is the tetravalent $ZrCl_4$ salt. The effect of $SmCl_3$ is similar to that produced by $CaCl_2$.

Two other non-group II divalent chloride salts were examined to determine if their behaviors were markedly different from the group II series. Fig. 3 *B* shows the results using $CdCl_2$ and $ZnCl_2$. Both these salts bring about small changes of the same type.

All of these results suggest that the interaction of an anion larger than a fluoride ion is required for the spectral change and that the cation modulates this change by a process that is related generally to its size rather than its charge. The optimal radius for the cation appears to be ~1 Å as inferred from the results of the two most effective ions, $CaCl_2$ (r = 0.99 Å) and $SmCl_3$ (r = 0.96 Å). From Figs. 1 *C*, 2 *B* and *C*, and 3 *A* and *B*, it can be seen that the maximum ellipticity change is reached by ~4 mM for all of the ions examined with a gramicidin concentration of 1 mg/ml. Consequently, the changes in ellipticity at 4 mM, 6 mM, and 8 mM for each ion were averaged to yield a more representative value for the maximum change in ellipticity produced by these cation chloride salts. Plotting these averaged values against the ionic radii of the cations (Fig. 3 *C*) highlights the possible link between the cationic radius and the ability of the cation to regulate the conversion process. All of the salts tested, except for $CdCl_2$, followed this general pattern.



DISCUSSION

In methanol and other organic solvents in the absence of ions, gramicidin exists in equilibrium as four different interwound double-helical conformers that differ in the orientation, handedness of the strands, and the stagger between the two monomers within the dimer (Veatch et al., 1974; Bystrov and Arseniev, 1988). All have hydrogen bonds in a β-sheet-type pattern and all have 5.6 residues per turn. The four types of double helices have been designated as follows: species 1, a left-handed parallel double helix with no stagger between the monomers; species 2, a left-handed out-of-register parallel double helix; species 3, a left-handed in-register antiparallel double helix; and species 4, a right-handed in-register parallel double helix. Although the spectra of isolated species 1 and 2 are similar, they differ in the relative magnitudes of the 229-nm and 210-nm peaks. The spectra of the other two species are very different (Veatch et al., 1974). The CD spectrum of gramicidin in methanol obtained in the absence of ions is a net spectrum due to the mixture of the four species (Wallace, 1983), weighted by the relative abundance of each species present in the solution.

The CD spectrum of gramicidin in the presence of multivalent cation salts (Fig. 1 *A*) strongly resembles the individual spectrum of species 1 (Veatch et al., 1974). Thus the titration appears to shift the equilibrium from a mixture of species in the absence of ions toward a single conformation similar to species 1, in the presence of multivalent cations. This is consistent with NMR observations that a single conformer exists after titration with high concentration of Ca^{2+} (Chen and Wallace, 1997). The structure of the species of gramicidin present in methanol in the presence of excess $CaCl_2$ has been determined by 2D ^{1}H-NMR (Chen et al., 1996). The structure is a left-handed parallel double helix with no stagger between the monomers (Fig. 4) and has been referred to as species 1′, as it is a slightly modified version of species 1 as defined by Veatch et al. (1974). It has been suggested from electrostatic calculations that the binding site for the divalent cations is produced by the carbonyl groups of the glycine residues at position 2 on each polypeptide chain. Only in the unstaggered parallel double-helical species 1, not in the antiparallel or staggered forms, are these two carbonyl groups at the same end of the dimer, and in close juxtaposition. Their geometry is such that a calcium ion would fit optimally in the cavity formed (Chen et al., 1996). Thus it could be that the cation fixes a conformation very similar to that of species 1 by forming a plug close to the end of the N-termini, with the anion binding possible at the opposite end of the double helix (which has a net positive charge). As SrF_2 has no effect on

FIGURE 1 Titration of the CD spectra of gramicidin upon the addition of the group II chloride salts. (*A*) Overlaid CD spectra of a 1 mg/ml gramicidin solution in methanol with increasing $SrCl_2$ concentrations. (*B*) Plots of the changes in ellipticity of the 229-, 210-, and 195-nm peaks as a function of $SrCl_2$ concentration. (*C*) Plot of the change in ellipticity at 229 nm upon the addition of different group II chloride salts.

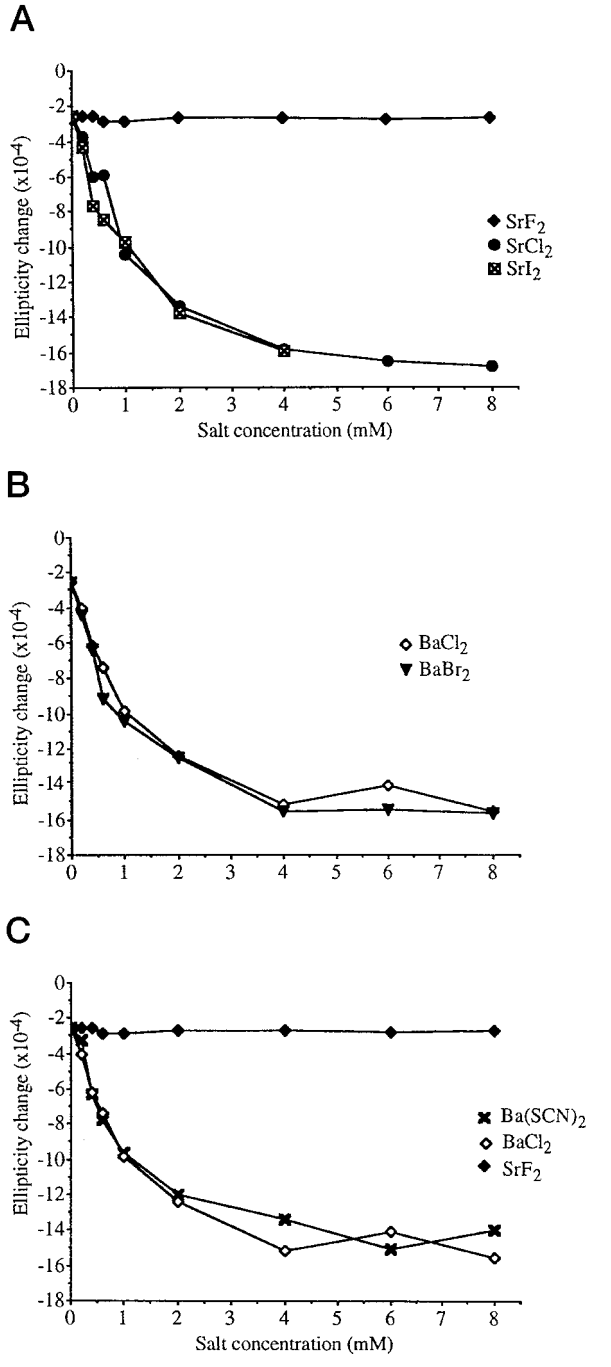


FIGURE 2 Changes in ellipticity at 229 nm of a gramicidin solution upon the addition of group II salts with different anions. (*A*) Addition of a series of strontium halides. Note that the strontium iodide-containing samples were only measured up to a concentration of 4 mM, as the strong optical absorption of this salt above that concentration made the measurements unreliable. (*B*) Addition of increasing concentrations of barium chloride salts. (*C*) Addition of barium thiocyanate, barium chloride, and strontium fluoride.

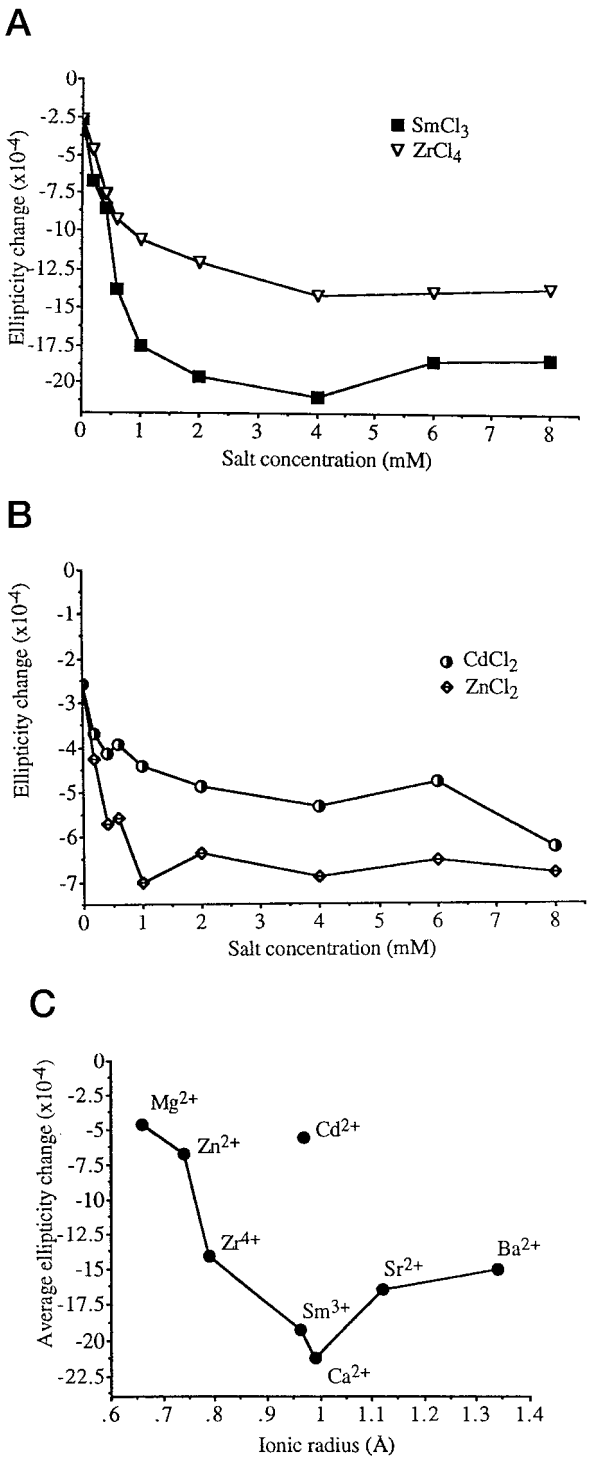


FIGURE 3 Changes in ellipticity of a gramicidin solution upon the addition of multivalent cationic chloride salts: plots of the change in ellipticity at 229 nm with increasing (*A*) samarium chloride and zirconium chloride and (*B*) cadmium chloride and zinc chloride. (*C*) Plot of the maximum ellipticity change at 229 nm for a number of multivalent chloride salts as a function of the Pauling radius.

the CD spectral change, whereas $SrCl_2$ and SrI_2 produce similar outcomes, this points toward a process that requires both a cation and an anion (Fig. 2 *A*). The cation and anion plugs of appropriate sizes could stabilize species 1′, thus shifting the normal equilibrium that exists between gramicidin dimers in a methanol solution. The scheme shown in Fig. 5 is proposed to illustrate this shift in equilibrium upon the addition of multivalent cationic salts.

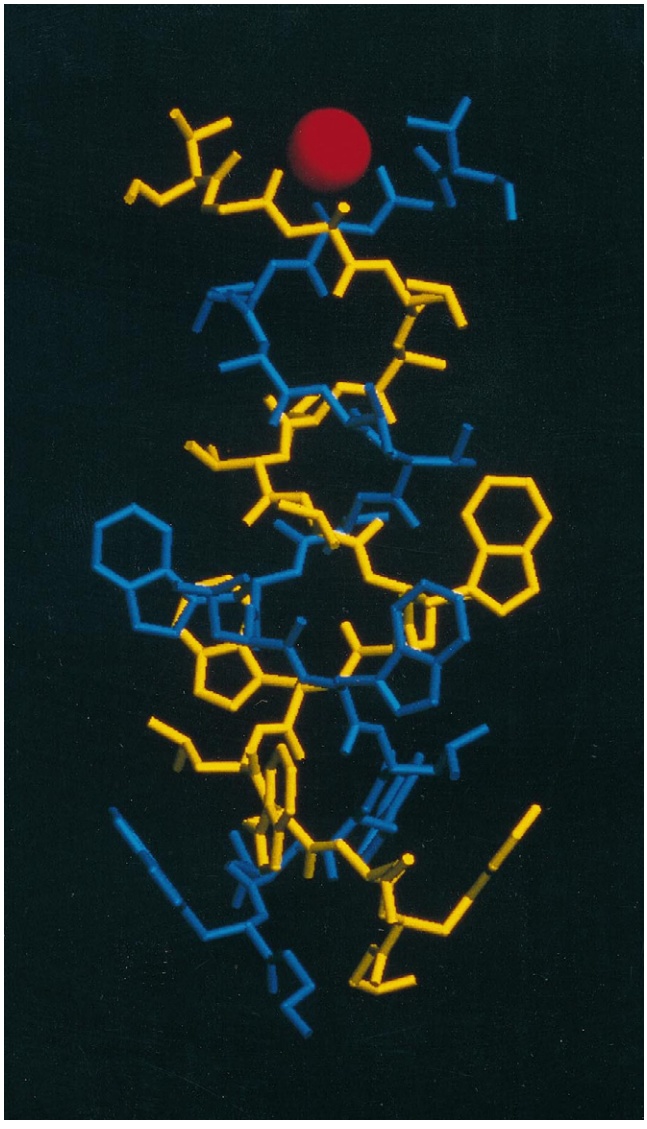


FIGURE 4 The gramicidin/calcium complex (coordinates [PDB accession code 1MIC] from Chen et al., 1996), with the proposed position of the divalent cation shown as a red circle.

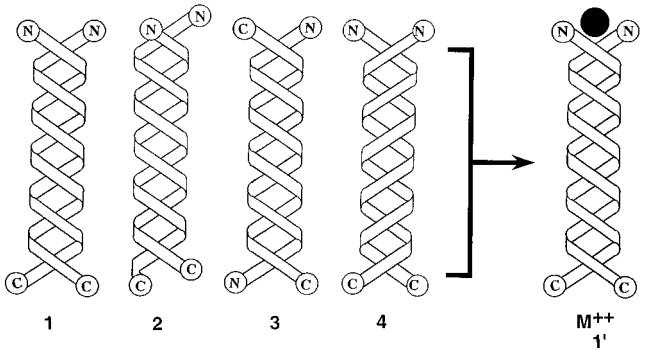


FIGURE 5 Schematic diagram showing the four species present at equilibrium in the ion-free solution, and their conversion into a single stable species in the presence of multivalent cations.

The charge on the multivalent cations was discounted as the cause of the spectral shift, as there was no correlation between the charge of the cation and the magnitude of the ellipticity change at 229 nm (Fig. 3). Then the differences in ionic radii of the cations were investigated (Fig. 3 *C*). These studies indicated that cations with an ionic radius similar to that of Ca^{2+} caused the biggest shifts in the CD spectrum. Cd^{2+}, however, did not follow this trend. One factor that can influence the radius of an ion is the coordination number. Generally, the greater the coordination number, the larger the effective ionic radius (r_e). However, the r_e still follows the Pauling ionic radius, with the larger ions having the larger r_e, e.g., for Ca^{2+}, r_e = 1.00 Å with the coordination number of 6 (co6), Sr^{2+} (co6) r_e = 1.18 Å, and Mg^{2+} (co6) r_e = 0.72 Å. The difference between the sizes for the smallest and largest coordination numbers for each cation is ~0.3 Å for all of the ions tested [Mg^{2+} (co8–co4) 0.32 Å, Ca^{2+} (co12-co6) 0.34 Å, Sr^{2+} (co12–co8) 0.26 Å, Ba^{2+} (co12–co6) 0.26 Å, Zn^{2+} (co8–co4) 0.30 Å, Sm^{3+} (co12–co6) 0.28 Å, Zr^{4+} (co9–co4) 0.30 Å], except for Cd^{2+} (co12–co4), which has a difference value of 0.53 Å. This may explain the anomalous behavior of Cd^{2+} in Fig. 3 *C*. If Cd^{2+} has a coordination number of 4 (r_e = 0.78 Å), this would place Cd^{2+} in a region more consistent with the other ions in Fig. 3 *C*. Of course, the true coordination numbers of Cd^{2+} and all of the other ions in these experiments are unknown, meaning that because the radii differences between the largest and smallest coordination states are approximately equivalent, the graph in Fig. 3 *C* can be moved from side to side within the boundary of 0.3 Å.

In conclusion, the equilibrium ensemble of species of gramicidin in methanol is shifted to a modified version of species 1 (species 1′) with the addition of multivalent cation salts. The mechanism proposed to explain this conversion assumes that all of the multivalent cation salts interact with the same binding site on species 1′, thus stabilizing this form, but not entering the pore. Assuming that all of the salts bind to the same location on species 1′, the variations with cation type in the magnitudes of the CD signals at the maximum salt concentrations may be due to the ions having different abilities to stabilize species 1′. The differing abilities of the various salts to stabilize species 1′ can be explained if they have different propensities for binding at this site. As for what the property of Ca^{2+} is that makes it the most suitable for this process, the physical size of the ion and not its charge appears to be the likely candidate. Hence cations with radii close to that of Ca^{2+} (0.99 Å) are best suited physically to interaction with the binding site, and most efficient in shifting the equilibrium mixture of double helices toward species 1′.

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