

THE TEMPERATURE DEPENDENCE OF GRAMICIDIN CONFORMATIONAL STATES IN OCTANOL

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In lipid bilayers and organic solvents, the hydrophobic polypeptide gramicidin adopts a number of different conformations, some of which are capable of conducting monovalent cations across phospholipid membranes. The equilibria between conformations have been shown to be influenced by factors such as lipid chain length, solvent, concentration and salt. In this study, the temperature dependence of the equilibrium mixture of double helical ion-free gramicidin in octanol was examined using circular dichroism spectroscopy.

INTRODUCTION

Gramicidin is a hydrophobic linear polypeptide antibiotic capable of forming ion channels in phospholipid membranes. It has the amino acid sequence: formyl-Val-Gly-Ala-DLeu-Ala-DVal-Val-DVal-Trp-DLeu-Trp-DLeu-Trp-DLeu-Trp-ethanolamine [1]. The unusual pattern of alternating L- and D-amino acids allows gramicidin to coil into helices with \pm -torsion angles (designated \pm -helices) with all their sidechains extending from the outer surface of a cylindrically-shaped molecule [2,3]. The alternating L-, D-sequence is also thought to be responsible for gramicidin's polymorphism [4]: it is capable of forming both helical dimer (HD) and double helical (DH) conformations, the former being the conducting form predominating in lipid bilayers, the latter being primarily found in organic solvents.

In many organic solvents, gramicidin forms a number of different types of DH structures of different hands, orientations and staggers [5]. In these types of structures, two monomers form interstrand hydrogen bonds aligned in either a parallel or antiparallel manner and then coil into helices. Under ion-free conditions, four such conformations have been detected thus far (Figure 1): species 1, which is a left-

handed helix with a parallel arrangement of its two chains, species 2 which is the same as species 1 but with a different stagger to its chains, species 3 which is a left-handed helix with an antiparallel arrangement of its chains, and species 4 which is a right-handed helix with parallel chains [3]. These conformations are interconvertible and exist in equilibrium with each other [6]. Varying conditions, such as solvent type [6,7], the presence of different salts [8,9], and peptide concentration [6] can drive the equilibrium to slightly favour one conformation over the others. The four species can be separated chromatographically in dioxane but on standing, will convert back to the equilibrium mixture [6]. The structures of various DH species have been examined in detail by NMR spectroscopy [10-13] and X-ray crystallography [14-18].

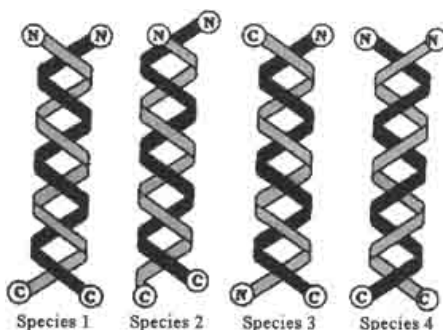


Figure 1. Schematic representation of the four types of ion-free DH conformations found for gramicidin in organic solvents (adapted from [9]).

Species 1, 3 and 4 produce distinct circular dichroism (CD) spectra [3]. The spectra of species 1 and 2, however, are so similar that they cannot be distinguished using CD spectroscopy. As species 4 differs from species 1 only in its helix sense, its CD spectrum is nearly the mirror image of species 1 (and 2). The relative abundances of the components of a mixture of these species can be quantitated by deconvolution of the CD spectrum of the mixture [7]. Solvent effects on the equilibrium have been systematically studied using alcohols of different chain length (from ethanol to dodecanol) [7]. It was found that increasing the chain length of the solvent (which consequently reduces solvent polarity) caused the relative abundance of species 1 & 2 to decrease from 41% to 22%, species 3 to increase from 57% to 68% and species 4 to increase from 2% to 10%. Species 3 dominates in all these solvents, but increases proportionately as the solvent polarity decreases. This is probably because being antiparallel, it is the only one of the species with no macrodipole [7].

Although mixtures of conformers are present in all of these solutions, to date all ion-free crystals produced (i.e. from methanol, ethanol, and propanol) have only been of species 3 conformations [15,18]. Therefore, it would appear that species 3 is the most stable DH conformation under a range of crystallisation conditions. This is not surprising as species 3 [15] has more hydrogen bonds than species 1 [11] and species 4 [12].

The goal of the present study was to determine whether variation in temperature could be used to drive the equilibrium of gramicidin DH conformations towards a single conformation, and to better understand the physical basis of the interconversion between the species. In addition, it was thought that this might aid with the production of crystals of the other species for high-resolution structural analyses.

Methods & Materials

Sample Preparations

Gramicidin D (ICN Biomedicals Inc.) was dissolved at room temperature in *n*-octanol (BDH Lab Supplies, 99% pure) at a concentration of 1 mg/ml and spun to remove any insoluble material. The sample was incubated at 5°C for 12 hours to allow for equilibration of the gramicidin conformations. Three independent preparations were examined.

CD Data Collection

A fixed pathlength (0.01 cm) Suprasil cell was used for all data collection and was sealed to prevent solvent evaporation at high temperatures. At each temperature, data were collected at intervals of 0.2 nm over a wavelength range of 275 nm to 190 nm using an Aviv 215 spectropolarimeter. Each scan was repeated three times. The baseline scan of the *n*-octanol solvent was performed only at 25°C as its CD spectra were not affected by temperature. The sample was examined over an increasing temperature range from 5°C to 65°C, at intervals of 10°C. After each increase in temperature, the sample was allowed to equilibrate for 15 minutes before spectra were obtained at that temperature.

CD Data Analyses

For each temperature, three scans were averaged and the baseline subtracted prior to a Savitsky-Golay filter being applied [19]. The spectra were deconvoluted using a linear least squares method in the program Super3 [20], with a reference set derived from the individual species spectra identified in 2-propanol by Veatch *et al.* [3], according to the method described by Chen and Wallace [7]. The experimental spectra were shifted to longer wavelengths by 1 nm to account for the different dielectric properties of *n*-octanol relative to 2-propanol, as it has been shown systematically for gramicidin that such a spectral solvent shift occurs [7].

A normalised root mean square deviation (NRMSD) parameter [20] was calculated as an indication of how well the back-calculated spectra produced by the least squares method fit with the experimental spectra. The lower the value, the better the fit and hence the more reliable representation of the conformations present in the sample. The algorithm used did not constrain the total of the fractions to sum to 1.0, so the Relative Total (RT) value calculated is an indication of the amount of peptide present in the sample that is producing a net signal.

RESULTS

The spectra of gramicidin in *n*-octanol between 5°C and 65°C all have roughly comparable characteristics: a broad negative peak at 225 nm with a shoulder at 220 nm and a narrower, more intense positive peak at 194 nm (Figure 2). As the temperature increases, there are decreases in the magnitudes of both peaks. The relative extent to which they decrease differs for the two peaks, resulting in a change in the ratio between the two peaks, and thus in the shape of the spectra between 5°C and 65°C.

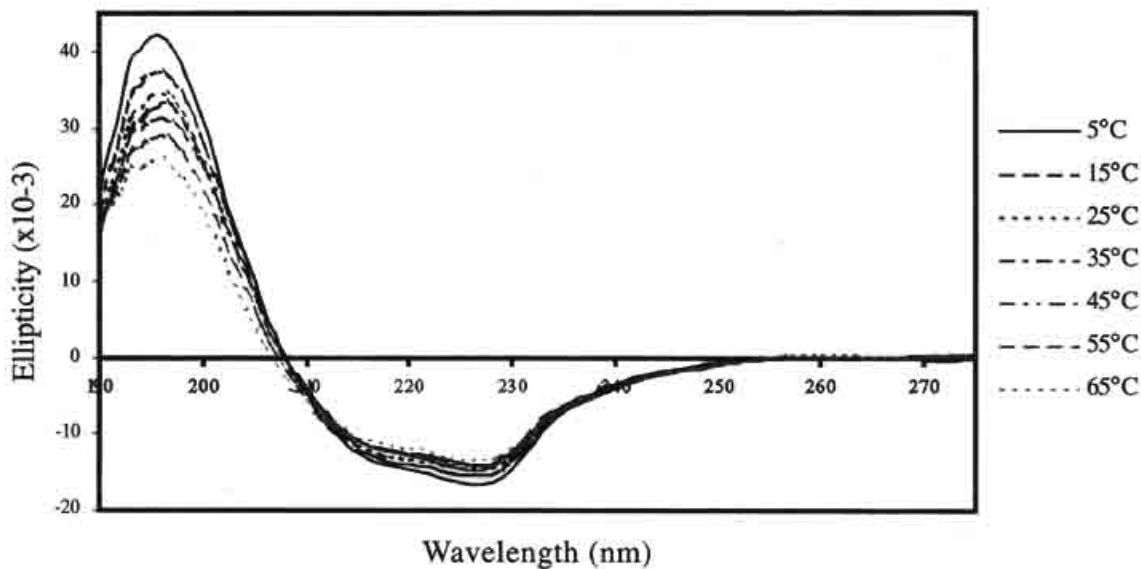


Figure 2. CD spectra of ion-free gramicidin in *n*-octanol over a temperature range of 5 °C to 65 °C.

The spectra between 5°C and 45°C share an isosbestic point at 209 nm, whereas in the spectra at 55°C and 65°C, the comparable region of the spectrum is shifted slightly to 211 nm. A shift in this point often indicates the appearance of an additional conformation.

According to the least squares analyses, between 5°C and 45°C there is no significant change in the calculated composition of gramicidin conformers (Table 1), despite the changes in peak magnitude at these temperatures. At 55°C and above, however, a dramatic change can be seen, where the abundance of species 1 & 2 increases and species 3 decreases accordingly.

The NRMSD values for the spectra between 5°C and 55°C are all ≤ 0.06 , indicating good fits between the experimental and calculated spectra and suggest that the reference set represents all of the conformations present in the sample. However, at 65°C the NRMSD value increases to 0.08, which suggests that there may be an additional conformation present at higher temperatures that is not represented in the reference set, consistent with the observed shift in isosbestic point.

Another feature to be noted is the change in the Relative Total. This value should remain constant and change only if there is a change in concentration, or if there is leakage, evaporation or precipitation (which will effect the concentration of the sample in the cell). In this experiment, the same sample was used across the whole temperature range, and no significant loss of sample single or precipitation were observed, even after returning to 5°C.

Table 1. The equilibrium compositions of gramicidin in *n*-octanol as a function of temperature calculated by least squares analyses.

Temperature (°C)	Species 1&2 (%)	Species 3 (%)	Species 4 (%)	RT	NRMSD
5	0	89	11	1.00	0.06
15	0	91	9	0.88	0.06
25	2	88	10	0.82	0.05
35	0	91	9	0.78	0.05
45	1	90	9	0.75	0.04
55	11	79	10	0.73	0.05
65	25	63	12	0.69	0.08

DISCUSSION

It is clear from the spectral data that the equilibrium composition of gramicidin conformations is dependent upon temperature, as the relative peak magnitudes change with temperature. However, there seem to be some discrepancies between the observed spectral data and the values calculated from the least squares analyses. Despite the spectral changes, the analyses suggest that there is no change in the composition between 5°C and 45°C.

The experimental CD spectrum of a peptide is the sum of all of its individual spectral components, weighted by their abundance in the sample. In the case of gramicidin, however, the reference set contains two spectra that are approximately mirror images of each other. As a result, the decrease in peak magnitudes observed could be the result of cancelling of equal amounts of these mirror image spectral components. Increases in the relative abundance of both mirror image components in the sample would cause a larger proportion of the total signal to be cancelled out and the net peak intensities to drop further.

The least squares analyses also suggest that the amount of peptide being detected in the sample is decreasing, even though there was no change in concentration or loss of sample during the experiments. It seems, therefore, that as a result of the cancelling effect, there is a proportion of the sample that is not being detected by the analyses. Thus, in order to provide a more accurate picture of the equilibrium composition, further analyses taking into account this undetected proportion, are required. Based on the RT values the compositional values can be determined. The undetected proportion must be approximately half species 1 & 2 and half species 4, so:

$$M = \frac{(1-RT) \times 100}{2}$$

where M is the missing % of species 1 & 2 and species 4 at that temperature.

The compositional values calculated from the least squares analysis can then be adjusted as follows:

$$\text{Species 1 \& 2} = (A \times RT) + M$$

$$\text{Species 3} = (A \times RT)$$

$$\text{Species 4} = (A \times RT) + M$$

where A is the abundance of the component calculated from the initial least squares analyses.

Table 2. Adjusted equilibrium composition values for gramicidin as a function of temperature, taking into account the undetected proportion.

Temperature (°C)	Species 1&2 (%)	Species 3 (%)	Species 4 (%)
5	0	89	11
15	8	80	14
25	11	72	17
35	11	70	19
45	14	67	19
55	21	58	21
65	33	43	24

The adjusted compositional values (Table 2) show that there is change in the equilibrium occurring at all temperatures, which is in agreement with the spectral observations. While the abundance of species 3 decreases, the abundances of both species 1 & 2 and species 4 increase.

Another factor to consider is the possible presence at higher temperatures of an additional component not represented in the reference set used. This is indicated by the increase in NRMSD at 65°C and the shift in isosbestic point. The additional component could be a monomer conformation, caused by

dissociation of the dimers at higher temperatures. In other analyses, monomers of gramicidin were assumed to be present only in negligible amounts [7]. Although no spectrum of “pure” monomer is currently available, the CD spectrum of the monomer is likely to be similar to that of the desformyl analogue of gramicidin, which cannot dimerise in lipid bilayers [21]. To test the possibility that the additional component is a monomer conformation, a spectrum was back-calculated using Super3, from the equilibrium composition calculated for gramicidin at 65°C in the least squares analysis. This spectrum is the expected representation of that equilibrium mixture, which was then subtracted from the observed experimental spectrum at 65°C. The difference between these two spectra should correspond to the component not represented in the reference set. The resulting difference spectrum was compared with the desformyl gramicidin spectrum. The two spectra were similar (data not shown), and completely different from those of any of the DH species 1 to 4. Both have a negative peak at long wavelengths, cross the x-axis at approximately 218 nm and have a positive peak above 210 nm. Small differences between the difference spectrum and the desformyl gramicidin spectrum may be attributed to the differences in the conditions used to obtain them (the latter was collected in lipid vesicles as opposed to octanol). The reference set used in the original least squares analyses was then edited to include the monomer spectrum derived from this study. Re-calculating the least squares analysis with this new reference set and adjusting the values as before, gave the calculated equilibrium composition values seen in Table 3. Between 5°C and 45°C there is no difference in the values compared to the previous analyses. At > 55°C, however, there appears to be some monomer present and the NRMSD values are significantly improved. This supports the idea that it is the monomer present at higher temperatures rather than unordered peptide.

Table 3. Equilibrium composition values for gramicidin as a function of temperature, calculated with the “monomer” spectrum incorporated into the reference set.

Temperature (°C)	Species 1&2 (%)	Species 3 (%)	Species 4 (%)	Monomer (%)	NRMSD
5	0	89	11	0	0.05
15	8	80	14	0	0.05
25	11	72	17	0	0.04
35	11	70	19	0	0.05
45	14	67	19	0	0.04
55	26	52	17	4	0.03
65	37	35	22	6	0.04

CONCLUSIONS

The overall trend for the temperature dependence of gramicidin in octanol from 5°C to 65°C is a very dramatic decrease in species 3 from 89% to 35%, a dramatic increase in species 1 & 2 from 0 to 37%

and a smaller increase in species 4 from 11% to 22%. While a large shift in composition is seen between 5°C and 65°C with a significant decrease in the abundance of species 3, the equilibrium composition was not driven to a single conformation. At the highest temperature, the proportions of all the DH species are nearly equal.

Species 3-type conformations are most often the dominant conformation in solution and are the only type of conformation yet seen in ion-free crystal structures. This suggests that this is the most stable DH conformation in solution, probably as a result of its antiparallel nature and it's having the most intermolecular hydrogen bonds. The results of this study are in agreement with this idea, since when the temperature increases, the equilibrium shifts away from species 3 and towards the suggested higher energy conformations. It is interesting to note that it is the left handed species 1 & 2, but not right handed species 4 that increases the most.

This study also supports the idea that the gramicidin monomers do have structure and that their CD spectral shape is similar to those seen for desformyl gramicidin. Although the monomer component is usually small, the new reference set created here, which includes a representation for monomers, may be useful in subsequent least squares analyses of other gramicidin samples.

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