The Aggregation of Gramicidin A in Solution†

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ABSTRACT: The kinetic and equilibrium constants relating monomer with four aggregated gramicidin species have been determined. These solution data were obtained by circular dichroism, fluorescence intensity, fluorescence polarization, and quantitative thin-layer chromatography measurements. Decreasing solvent polarity strongly favors the aggregated species, and the decay times of these species are sufficiently long for them to be physically isolated. The measured concentration dependence of the rate of forward aggregation proves that a dimerization is the rate-limiting step. Evidence is presented suggesting that all of the gramicidin isolated species (each of which has a distinct conformation) are dimers. Measurements of the relative proportions of the gramicidin isolated species at equilibrium show that substitution of a methyl group for the gramicidin formyl proton does not affect the equilibrium parameters of any of the isolated species.

In the preceding paper (Veatch *et al.*, 1974), the isolation of four conformational species of gramicidin was described. These species, isolated from a single nonpolar solvent system, are denoted 1, 2, 3, and 4 in order of increasing mobility on thin-layer chromatography (tlc). It was concluded that species 1, 2, and 4 are probably helical structures with largely parallel-β hydrogen bonding; species 1 and 2 have a handedness opposite that of species 4. Species 3 has largely antiparallel-β hydrogen bonding.

The work presented here is qualitatively consistent with previous osmometry measurements, suggesting that gramicidin dimerizes in nonpolar solvents (Sarges and Witkop, 1965; Isbell *et al.*, 1972). In view of the demonstrated conformational heterogeneity of the gramicidin isolated species (Veatch *et al.*, 1974), further equilibria among the various conformational species must be considered. Evidence is presented which supports the possibility that *all* of the isolated species are dimers.

The following parameters were measured in a single solvent: (1) the rates of disaggregation of each of the isolated species, (2) the total equilibrium dimerization constant, and (3) the relative proportions of the isolated species in equilibrium at high concentration. With decreasing solvent polarity, the disaggregation rates decrease dramatically. The observed concentration dependence of the rate of forward aggregation from monomer proves that a dimerization is the rate-limiting step. The parameters of gramicidins A, B, and C and *N*-acetyldesformylgramicidin are compared to determine the effect of structural modifications upon the stability of the isolated species in solution. Finally, what is known about the structure of the gramicidin transmembrane channel is discussed in terms of the features of the various structures in solution.

Experimental Section

Materials. Countercurrent purified gramicidins A, B, and C were kindly furnished by Dr. Erhard Gross of the National Institutes of Health. Unless otherwise specified, "gramicidin" refers to a once-crystallized (ethanol) mixture of gramicidin A (85%), gramicidin B (10%), and gramicidin C (5%) (a gift from S. B. Penick and Company). *N*-Acetyldesformylgramicidin was prepared from gramicidin by the procedure of Sarges and Witkop (1965). The ^{1}H nuclear magnetic resonance (nmr) spectrum of this analog in Me_2SO-d_6 lacked the formyl proton resonance of 8.1 ppm and had an acetyl methyl resonance at 1.9 ppm.

The solvents used were all spectroquality grade.

Fluorescence measurements were made with an Hitachi-Perkin Elmer MPF-2A spectrofluorimeter with temperature control and polarization accessories. Excitation was at 290 nm, and emission was monitored at 340 nm. Stoppered 1-cm quartz cells were used, and the temperature was controlled at 23°. Excitation and emission slits of 6 and 10 nm, respectively, were used for intensity measurements, but the emission slit was widened to 20 nm for the fluorescence polarization measurements. The polarization was calculated as $(R - 1)/(R + 2)$, where R is the intensity ratio of parallel to perpendicular after correction for the efficiency of the emission monochrometer grating.

In methanol the decay rates of the isolated species are too rapid to obtain accurate values by dilution into methanol; therefore, the equilibrated methanol samples were diluted into ethyl acetate–methanol (98:2). Similarly, the equilibrated dimethyl sulfoxide (Me_2SO) solutions were diluted into ethanol–Me_2SO (95:5). At concentrations between 10^{-5} and 10^{-7} M, it was important to subtract appropriate solvent blanks, and usually several sequential measurements on the same sample were averaged. For the longer equilibrations the samples were stored in glass volumetric flasks at room temperature. After a few days gramicidin A at low concentration in ethyl acetate began absorbing to the glass of the volumetric flasks.

Circular Dichroism Measurements. The concentration dependence of the ellipticity at 295 nm in ethanol was measured directly, using a Cary 61 (kindly made available by Dr. R. J. Kitz at the Massachusetts General Hospital). Cells of path lengths from 0.1 mm to 10 cm were used with the concentration determined by absorption assuming an extinction coefficient at 290 nm of 20,000 cm^{-1} M^{-1}. The

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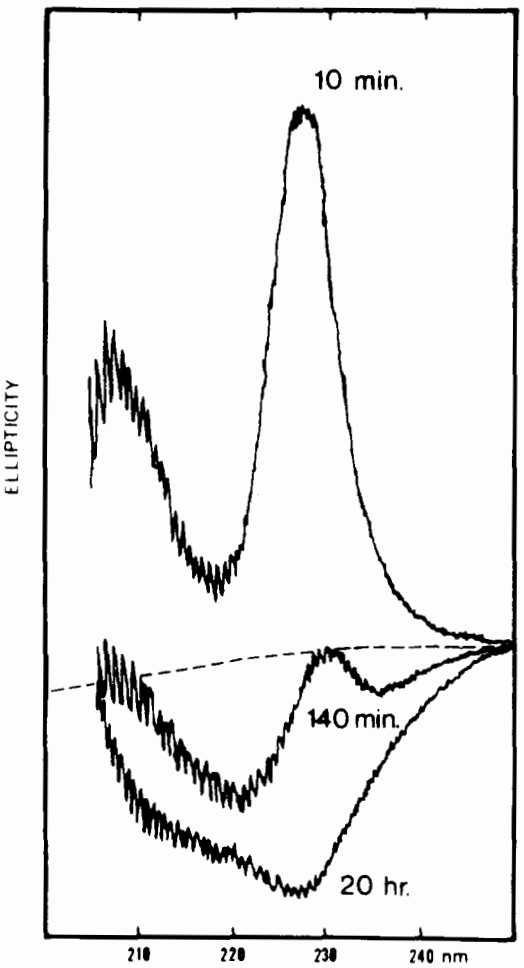

FIGURE 1: Time dependence of the CD spectrum of gramicidin isolated species 4 in ethanol. Species 4 was prepared using the minimum interconversion protocol described in the Experimental Section of the previous paper (Veatch *et al.*, 1974). At zero time an aliquot of species 4 in ethyl acetate was diluted 1/100 in ethanol to a final concentration of about 5×10^{-6} M in a 1-cm cell. The dashed line is the base line.

samples were equilibrated for over 15 hr at room temperature prior to measurement.

The circular dichroism (CD) spectra in the region 200 to 250 nm were recorded with a Cary 60 with Model 6001 CD attachment. For the decay experiment in ethanol a 1.0-cm cell was employed; for the measurements of ellipticities at 210 and 228 nm in dioxane, a 0.1-mm cell was used. Molar ellipticity was converted to residue ellipticity by dividing by 15.

Quantitative Elution from Thin-Layer Chromatograms. The gramicidin (or analog) sample was equilibrated at 5×10^{-2} M in ethanol, then diluted 1/100 into dioxane. Approximately 0.03 ml of this dioxane solution was banded near the bottom of a 5 × 20 cm silica tlc plate (Quantum, QIF) and dried rapidly with dry nitrogen. After the chromatogram was developed in dioxane–water (100:1), it was dried and the bands were visualized with ultraviolet light. The silica was divided up into strips, each containing one band, and the gramicidin was eluted quantitatively with 2 ml of ethanol. The concentration was obtained by measuring the absorbance at 290 nm with appropriate blank subtraction.

Vapor Phase Osmometry. A Perkin Elmer-Hitachi vapor pressure osmometer (kindly made available by Dr. Harold Simons of the Kendall Corporation) was used with 2-propanol (50°) and ethyl acetate (40°). Benzil (molecular weight 210 g/mol) was used as a standard. The gramicidin had been crystallized slowly from ethanol, dried under vacuum at 50° for 5hr, and was dissolved in the 2-propanol without heating.

TABLE I

a. Decay Half-times for Isolated Gramicidin Species at Low Concentration Obtained from Fluorescence Polarization, Fluorescence Intensity, and Ellipticity at 228 nm (23°)

Species	Ethanol	Ethanol–Ethyl Acetate (1:4)[a]
1 + 2	120 min	100 hr
3	30 min	8 hr
4	70 min	40 hr

b. Fluorescence Parameters of End States for Decay of Isolated Species at 10^{-6} M in Ethanol (23°)

Species	Polarization		Intensity Decrease (%)
	Initial	Final	
1	0.024	0.007	30
2	0.023	0.007	30
3	0.026	0.007	0
4	0.023	0.007	20

[a] Half-times from fluorescence polarization measurements only.

Results

Decay of the Isolated Species. When one of the gramicidin isolated species was dissolved in ethanol at a concentration below 10^{-5} M, any changes in the fluorescence polarization, fluorescence intensity, or ellipticity at 228 nm followed an exponential time course. For each species the disaggregation half-times deduced from the different parameters were the same within the accuracy of the measurements. Figure 1 shows the progressive changes in the CD spectrum of species 4 with time. The decay half-times for the isolated species, listed in Table Ia, are slow in ethanol and even slower in a less polar solvent. Note that the half-times for the species are changed little in relation to one another by the change in solvent polarity. In the following discussion it will be argued that disaggregation is the rate-limiting step which is monitored by these parameters, monomer conformational changes being more rapid.

The parameter values reached at equilibrium are a function of the concentration, as will be demonstrated (*vide infra*). In Table Ib values for the fluorescence polarization and intensity change at 1×10^{-6} M in ethanol are tabulated. Note that all of the species have about the same polarization,[1] but that species 3, the fastest decaying species, has no fluorescence intensity decrease upon decay. Species 1 and 2 interconvert with each other in ethyl acetate–ethanol (1:4) in about 0.5 hr when assayed on tlc, much faster than the decay of either to monomer as followed by fluorescence parameters.

Dimerization Equilibrium. Three parameters of gramicidin in ethanol are plotted as a function of concentration in Figure 2: (a) fluorescence polarization, (b) fluorescence in-

[1] However, the fluorescence polarization of species 3 in ethyl acetate (23°) is 0.019 for species 1, 2, and 4 and 0.025 for species 3, a significant difference.

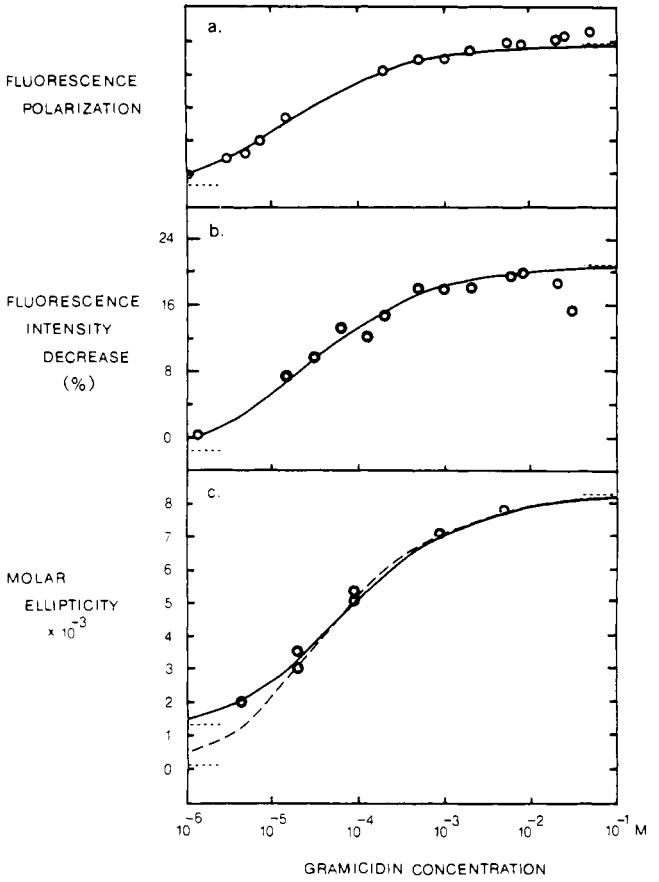

FIGURE 2: Concentration dependence of three gramicidin parameters at equilibrium in ethanol. All samples were equilibrated at room temperature at least 24 hr prior to measurement. The curves are theoretical dimerization curves fitted visually to the data points. The dotted lines are the fitted end points. The measurements are described in the text. (a) Fluorescence polarization; (b) fluorescence intensity; (c) molar ellipticity at 295 nm.

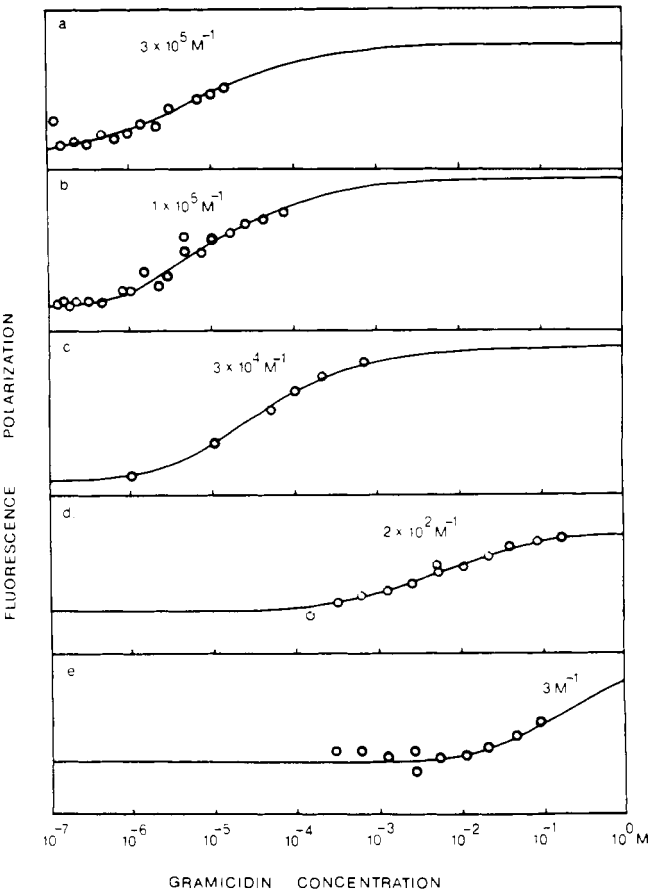

FIGURE 3: Concentration dependence of gramicidin fluorescence polarization at equilibrium in solvents of varying polarity. Gramicidin A was used for (a), (b), and (c), and gramicidin (85% gramicidin A) for (d) and (e). The polarization values are in arbitrary units. In dioxane (a) and ethyl acetate (b) solutions were made from gramicidin A, lyophilized from acetic acid, at the highest concentration, diluted serially, and stored in glass volumetrics. The lines are theoretical dimerization curves for the indicated K_2 values. The solvents and equilibration times are (a) ethyl acetate (2 days); (b) dioxane (35 days); (c) ethanol (1 day); (d) methanol (1 day); (e) Me_2SO (3 hr).

tensity, and (c) the CD ellipticity at 295 nm. The CD measurements were made directly on solutions at equilibrium using cells of varying path length. To avoid artifacts, all fluorescence polarization measurements were carried out at absorbances of less than 0.7 cm^{-1} ($\sim 3 \times 10^{-5}$ M). For higher concentrations the equilibrated samples were first diluted; the value of the polarization, extrapolated to the time of dilution, was used to characterize the high concentration equilibrium. Of course, only slowly decaying species, such as the isolated species, would be detected by this dilution method. The fluorescence intensity parameter is defined to be the per cent decrease in the intensity from immediately after dilution to the equilibrium (at 1×10^{-6} M) reached many hours later.

The expected equilibrium behavior for a simple monomer–dimer equilibrium is given in Appendix eq 5. All of the parameters are, in effect, normalized to the concentration. Hence, at any concentration the observed parameter value will be the sum of the dimer and monomer values, each weighted by its respective mole fraction.[2] The curves in Figure 2a–c are theoretical curves of the mole fraction in dimer as a function of the log of the total gramicidin concentration, fitted visually to the data points to determine the dimerization constant. Of course, an uncertainty in the parameter values for monomer and dimer alone contributes to the uncertainty in the estimated value of the dimerization constant, especially for the CD measurements which could not be carried out at very low concentration. The fitted dimerization constants are: 5×10^4 M^{-1} for the polarization (Figure 2a), 3×10^4 M^{-1} for the fluorescence intensity (Figure 2b), and 1×10^4 M^{-1} (dotted curve) to 2×10^4 M^{-1} (solid curve) for the ellipticity at 295 nm (Figure 2c). The average estimate is 3×10^4 M^{-1}. Since all dimers must be in constant ratio with one another at equilibrium, the observed dimerization constant represents a weighted sum including *all* dimers, even though the parameter measured may be a property of only a single dimer (see Appendix).

The solvent dependence of the dimerization constant was quantitated using fluorescence polarization measurements as shown in Figure 3. Because the absolute polarization values are dependent upon the tryptophan emission lifetime (which is itself dependent upon solvent polarity), the polarization scale is in arbitrary units. The dimerization constants from the fitted theoretical curves are shown in the figure and are tabulated in Table II. In ethyl acetate and dioxane it is unlikely that true equilibrium was obtained, since the decay rates were on the order of weeks. In Me_2SO the dimerization constant has been calculated by assuming that the species composition is the same as that obtained at

[2] This statement is true to a good approximation for the fluorescence polarization as long as the polarizations are small and the monomer and dimer have nearly the same quantum yields.

TABLE II: Summary of Rate and Equilibrium Data for Gramicidin Mixture in Solvents of Varying Polarity (23°).

Solvent	K_2 (M^{-1})	Average k_2^- (sec^{-1})	k_2^+ (M^{-1} sec^{-1})
Dimethyl sulfoxide	3		
Methanol	2×10^2	2×10^{-2}	(4)[b]
Ethanol	3×10^4	4×10^{-4}	(12)[b]
Ethyl acetate	3×10^5 [a]	$\approx 2 \times 10^{-6}$	2
Dioxane	1×10^5 [a]	$\approx 5 \times 10^{-7}$	

[a] Complete equilibrium probably not achieved. [b] Calculated values.

high concentration in ethanol. At 100 mg/ml (5×10^{-2} M) of methanol, the concentration used for the ^{13}C nmr measurements in the following paper (Fossel *et al.*, 1974), a dimerization constant of 2×10^2 M^{-1} implies that 80% of the gramicidin is in the dimer state. The same concentration in Me_2SO with a dimerization constant of 3 M^{-1} implies only 20–30% dimer.

Relative Proportions of the Isolated Species. The isolated species each run true on thin-layer chromatography. If gramicidin monomer is applied to the plate (gramicidin A lyophilized from acetic acid, *vide infra*), the effective concentration on the plate is so high that forward aggregation occurs very rapidly, yielding the various isolated species. Therefore, if tlc is to be used to quantitate the isolated species proportions, it is necessary to make the monomer mole fraction negligible.

In ethanol at a concentration of 5×10^{-2} M, the measured dimerization constant implies a monomer mole fraction of only 2%. In order to avoid interconversion upon application of the sample to the tlc plate, the equilibrated ethanol solution was diluted 1/50 with dioxane; after development and quantitative elution, the proportions shown in Table IIIa were found.

TABLE III: Estimation of the Relative Proportions of the Isolated Species at Equilibrium in Ethanol at 100 mg/ml (23°).

	Mole Fraction			
	1	2	3	4
a. Quantitative elution from thin-layer chromatogram	0.16	0.23	0.46	0.14
	1 + 2		3	4
b. Analysis of peptide CD in dioxane			0.46	0.42	0.13

c. Data used for CD calculations

Sample	Residue Ellipticity (deg cm^2 $dmol^{-1}$) 228 nm	210 nm
Species 4	+47,600	+21,200
Species 3	−26,800	−3,000
Species (1 + 2)	−28,400	−24,400
Gramicidin from ethanol	−18,100	−9,800

TABLE IV: Equilibrium Data for Gramicidin A, Gramicidin B, and *N*-Acetyldesformylgramicidin in Ethanol (23°).

		Species Mole Fraction (at 5×10^{-2} M)			
	K_2 (M^{-1})	1	2	3	4
Gramicidin A	3×10^4	0.17	0.27	0.41	0.14
Gramicidin B	1×10^5	0.22	0.14	0.59	0.05
Gramicidin C	3×10^4				
Gramicidin	3×10^4	0.16	0.23	0.46	0.14
N-Acetyldesformylgramicidin	2×10^4	0.12	0.30	0.44	0.14

Alternatively, measurement of the CD ellipticity at 210 and 228 nm of a similar dilution into dioxane can be used to solve algebraically for the mole fraction of species 4, species 3, and the sum of species 1 and 2, since the ellipticities of these species in dioxane are known (see Veatch *et al.*, 1974). The resulting proportions are listed in Table IIIb and agree well with the tlc results.

In ethyl acetate species 1 and 2 reach equilibrium with each other in a few days (as assayed on tlc), long before either yields species 3 and 4. Equilibration of species 1 and 2 at a range of concentrations showed the relative proportion of species 1 and 2 to be concentration independent, when assayed by tlc after concentration by evaporation. Concentration by evaporation in ethyl acetate did not in itself interconvert species 1 and 2. This partial equilibrium experiment suggests that species 1 and 2 involve the same number of gramicidin molecules.

Equilibrium Parameters for Some Gramicidin A Analogs. The observed dimerization constants (from fluorescence polarization measurements) and the proportions of each species at high concentration (from tlc) in ethanol are tabulated in Table IV for gramicidin, gramicidins A, B, and C, and *N*-acetyldesformylgramicidin.

Forward Rate Measurements. If monomer is introduced into a solvent at a concentration where almost all of the material would be dimer at equilibrium, then the kinetics of the monomer mole fraction would obey Appendix eq 10. The effective rate constant, $k_2^+[G_0]$, is proportional to $[G_0]$, the total gramicidin concentration, as well as to k_2^+, the forward dimerization rate constant (M^{-1} sec^{-1}). If a trimerization or a tetramerization were taking place instead of dimerization, then a square or cube dependence on $[G_0]$ would be expected.

When gramicidin A, lyophilized from acetic acid, was dissolved in ethyl acetate, the fluorescence polarization was found to be much lower than the values of the isolated species; in fact, it was even lower than the value obtained at low concentration in Figure 3a. The polarization increased nearly exponentially with time; the half-time at 5×10^{-6} M was 18 hr and at 5×10^{-5} M was 2.1 hr (23°). This first power dependence strongly implies a dimerization as the rate-limiting step. Thus, the lyophilized gramicidin A appears to be monomer gramicidin if the aggregated state is dimer. The appropriate kinetic plot is shown in Figure 4, yielding a forward rate constant of 2.0 M^{-1} sec^{-1} (Table II).

Vapor Pressure Osmometry on Species 3. Table V shows the apparent molecular weights obtained for gramicidin crystals dissolved in 2-propanol as a function of concentra-

tion. Although the measurements were made at 50°, the sample reservoir was at room temperature; species 3 is stable at 50° in 2-propanol for at least 1 hr. Extrapolation to zero concentration yields a molecular weight of 4600 g/mol (compared to monomer = 1880; dimer = 3760; and trimer = 5640 g/mol). It is not obvious why the apparent molecular weight appears to *decrease* to 3800 g/mol at the highest concentration. A value of 4600 g/mol was also obtained at 2 mg/ml in ethyl acetate at 40°.

Discussion

Consistency of Rates and Equilibria with Dimerization Model. The quantitative concentration dependence of the kinetics of forward aggregation proves that an aggregation of the form, $2G_n \rightarrow G_{2n}$, is rate limiting. The fit of the theoretical dimerization curve to equilibrium data implies an aggregation of the same form in ethanol. Since previous osmometry results (Sarges and Witkop, 1965; Isbell *et al.*, 1972) heavily favor a dimer molecular weight over a tetramer at high concentration in these solvents, it is almost certain that $n = 1$, and to a first approximation, we are dealing with a monomer–dimer system. Hence, the low polarization state achieved at low concentration (or with gramicidin A lyophilized from acetic acid) must be monomer.

The forward aggregation kinetics suggest, but do not prove, that for gramicidin monomer conformational changes are rapid relative to the rates of aggregation and disaggregation.[3] In this case, the exponential decay rate for each of the isolated species corresponds to the disaggregation rate for that species.[4]

For a simple dimerization the equilibrium constant would be equal to the forward rate constant divided by the decay constant. This is approximately true for the measurements in ethyl acetate tabulated in Table IV. In view of the conformational heterogeneity of the system and the absence of equilibrium, this agreement is expected only to within an order of magnitude.

Solvent Dependence of Rates and Dimerization Constant. Table IV shows the solvent dependence of the dimerization constant, K_2, and the average decay rate,[5] k_2^-. An estimated forward rate, k_2^+, has been calculated for methanol and ethanol as the product of K_2 and k_2^-. The large changes in the dimerization constant are largely due to decreases in the decay rate. This dramatic kinetic and equilibrium stabilization in nonpolar solvents strongly suggests dimers with a large number of intermolecular hydrogen bonds, such as the double helical dimer structures described in the preceding paper. The interconversion of species 1 and 2 (as followed by tlc) is more than an order of magnitude faster than the decay of either species 1 or 2 (as followed by spectroscopic parameters).

The very slow aggregation rates of the isolated species in dioxane and ethyl acetate make equilibrium measurements very difficult. A lack of equilibrium would introduce large errors into the calculation by Isbell *et al.* (1972) of dimerization constants from osmometry data in these solvents (and perhaps in 1-propanol as well). The concentration dependence of the CD and infrared spectra of gramicidin reported by Isbell *et al.* (1972) cannot be interpreted in terms of a dimerization equilibrium. As the characterization of the gramicidin isolated species in the previous paper (Veatch *et al.*, 1974) graphically demonstrates, in the absence of equilibrium the sample history must be carefully specified for the results to be reproducible, a factor not considered by Isbell *et al.* (1972). As indicated in the experimental section, for ethanol and methanol solutions equilibrium is reached within a few hours at room temperature. Is-

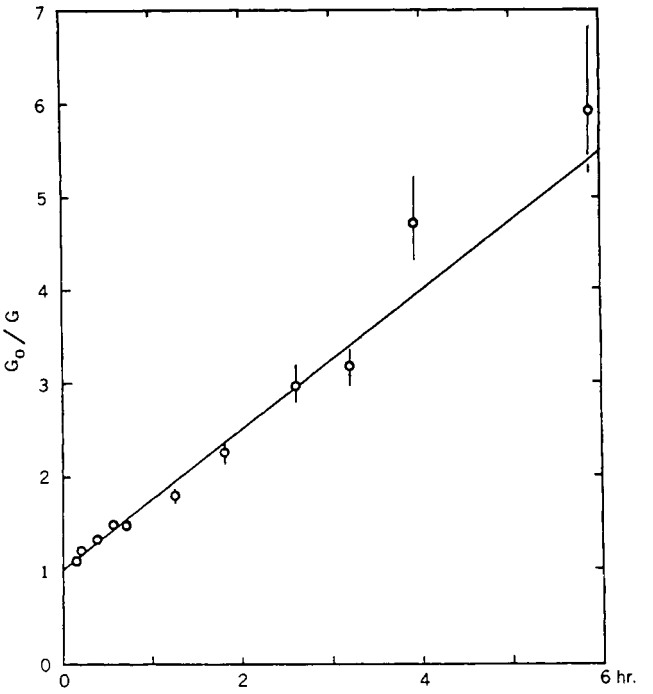

FIGURE 4: Forward dimerization kinetics for gramicidin in ethyl acetate. Gramicidin A lyophilized from acetic acid was dissolved in ethyl acetate at a concentration of 5×10^{-5} M at 23°. The inverse of the free monomer mole fraction, $(G_1/G_0)^{-1}$, is plotted here as a function of time. The monomer mole fraction was taken to be proportional to the fluorescence polarization at very long times minus the fluorescence polarization at the indicated time; further, the intercept on the vertical axis has been assumed to be $(G_1/G_0) = 1$. The error bars denote only relative error. Correction of the mole fraction for a 15% decrease in fluorescence intensity with aggregation did not alter the plot. The slope corresponds to a forward rate of 2.0 M^{-1} sec^{-1}.

TABLE V: Vapor Pressure Osmometry Data for Gramicidin Isolated Species 3.

Solvent	Gramicidin Concn (mg/ml)	Number-Average Molecular Weight (g/mol)
2-Propanol	9.9	4600
	20.2	4200
	30.2	4000
	39.6	3800
	Weighted av	4100
	Extrapolated to zero concentration	4600
Ethyl acetate	1.95	4500

[3] It is possible that the monomer was already locked into a proper conformation for aggregation in the forward rate experiment.

[4] The single exponential decay rate for fluorescence polarization (which probably monitors aggregation) and ellipticity at 228 nm (which probably monitors conformation) is also consistent with rate-limiting aggregation. For the double helical dimers, postulated in the previous paper (Veatch *et al.*, 1974), aggregation would require a large concerted conformational change.

[5] This rate for ethyl acetate and ethanol is one taken from the semilog plot of fluorescence polarization for a mixture of the isolated species; in methanol the fluorescence intensity was used.

bell *et al.* (1972) calculated a dimerization constant of 5×10^1 M^{-1} in methanol and 5×10^2 M^{-1} in ethanol (at 30°) from their osmometry data, compared with 2×10^2 M^{-1} in methanol and 3×10^4 M^{-1} in ethanol (at 23°) found by us. It is unlikely that the temperature dependence of the dimerization constant could account for the two orders of magnitude difference in ethanol. The osmometry data are most sensitive to errors due to nonideality of the component monomers and dimers, such as that observed for isolated species 3 in 2-propanol.

Evidence of the State of Aggregation of the Individual Isolated Species. At 5×10^{-2} M in ethanol the measured dimerization constant implies about 2% monomer; therefore, since all of the isolated species are present at greater than 10%, none of them can be monomer. The concentration profile in Figure 2a shows a slight upward deviation from the theoretical dimerization curve at higher concentration, which could possibly be a sign of higher order aggregation. This profile largely measures the disappearance of monomer and would not be very sensitive to higher aggregates with properties similar to the dimers. A short-lived higher aggregate could also not be excluded if it decayed rapidly to dimers.

The forward rate measurements imply that at least the fastest aggregating species, probably species 3,[6] is a dimer. The osmometry results are equally consistent with species 3 being a dimer or a trimer. Since X-ray diffraction data on the crystals from which species 3 is obtained have revealed a dimer in the asymmetric unit and two asymmetric units in the unit cell (see discussion in the previous paper, Veatch *et al.*, 1974), it is likely that species 3 is a monomer, a dimer, or a tetramer, but *not* a trimer. Therefore, a dimer structure is strongly favored for species 3.

The fluorescence intensity profile in Figure 2b implies that one or more of species 1, 2, and 4 must be a dimer, since species 3 has no intensity change. The positive ^1L_b bands associated with species 1 and 2 (see Veatch *et al.*, 1974) also appear to follow the dimerization (Figure 2c). Although it is tempting to suggest that species 1 and 2 are a dimer-tetramer pair, since their conformational properties are so similar, the partial equilibrium experiment suggests that they have the same molecularity, presumably that of a dimer.

Since species 4 is present at less than 15% of the total, it is not possible to draw conclusions about its molecularity from measurements of equilibrium parameters for which the other species present have similar values. However, since it has a CD spectrum which is an approximate mirror image to that of species 1 and 2, both of which are probably dimers, it is certainly possible that it is a dimer also.

Nature of the Monomer State. The monomer state at low concentration in ethanol has low fluorescence polarization and no ^1L_a aromatic CD band, while the isolated species all have higher polarization and a positive ^1L_a band. These changes could be due to increased flexibility in the monomer state; however, the decrease in fluorescence polarization could also be due to smaller size. Definitive data on the increased flexibility of the monomer conformation(s) is obtained from the ^{13}C nmr relaxation measurements in monomer and dimer solvents (see following paper, Fossel *et al.*, 1974).

Comparison of Equilibrium Parameters of Gramicidin Analogs. Table IV tabulates the dimerization constant and the proportions of the isolated species at equilibrium in ethanol for the natural gramicidin analogs and the synthetic *N*-acetyldesformylgramicidin. (It is assumed that the analog structures are isomorphous with those of gramicidin A and have the same relative tlc mobilities.) Note first that gramicidin (which is 85% gramicidin A, 10% gramicidin B, and 5% gramicidin C) has approximately identical values with gramicidin A. This justifies the use of gramicidin for the conformational study of the isolated species in the previous paper (Veatch *et al.*, 1974).

The replacement of the formyl proton in gramicidin by a methyl group in *N*-acetyldesformylgramicidin has not significantly altered either the dimerization constant or the species mole fractions. Independent of assumptions about whether any given isolated species is a dimer, trimer, tetramer, etc., this observed invariance implies that none of the isolated species is energetically destabilized relative to the average monomer conformation in ethanol. Urry (1971) has argued that this substitution would destabilize the head-to-head π(LD) helical dimers in the hydrophobic membrane interior environment due to the steric hindrance of the two methyl groups and the resulting disruption of the interhelix hydrogen bonding pattern. The absence of destabilization by as much as a factor of 2 for *any* of the isolated species suggests that none is a head-to-head π(LD) helical dimer (tail-to-tail and, perhaps, head-to-tail dimers would still be consistent with these data); however, the degree of destabilization to be expected in ethanol solution is less clear (*vide infra*).

Countercurrent purified gramicidin B differs from gramicidin A in having a dimerization constant larger by a factor of 3, an average decay rate decreased by a factor of 3, and species 1 and 3 increased relative to species 2 and 4. If it is assumed that *all* of the isolated species are, in fact, dimers, then the effective dimerization constant for each species is equal to the product of the observed dimerization constant and the species mole fraction (see Appendix). Such a calculation with the data in Table IV suggests that the substitution of the phenylalanine for tryptophan at residue 11 increases the effective dimerization constant of species 1 and 3 several fold, while leaving those for species 2 and 4 unchanged. (The observed dimerization constant of a somewhat impure sample of gramicidin C was the same as that of gramicidin A.)

Relationship between the Structure of the Gramicidin Transmembrane Channel and Those of the Gramicidin Isolated Species. Evidence has been presented that the transmembrane channel is an ion-conducting dimer in rather slow equilibrium with nonconducting monomer (Bamberg and Läuger, 1973). Some, if not all, of the gramicidin isolated species are dimers in slow equilibrium with monomer.

The channel probably contains some water (Myers and Haydon, 1972) and is less than 30 Å in length (Hladky and Haydon, 1972). The only direct estimate of the dimensions of any of the isolated species is for species 3; two of three possible dimensions suggested by the crystallographic data are cylinders 27–32 Å in length (see discussion in Veatch *et al.*, 1974). There is no direct evidence that any of the gramicidin isolated species have internal cavities, though the conclusion that species 1, 2, and 4 are helical implies at least a small hole down the middle. The data for species 3, which support an antiparallel-β double helix, also imply a substantial cylindrical hole since this model cannot be made without one.

[6] Species 3 has the fastest decay rate and is present in the largest amount of the isolated species in ethanol; hence, it would have to have the fastest forward rate in ethanol if all of the species are dimers.

Although it has been postulated that the transmembrane channel dimer involves a left-handed helix and is symmetric (Urry, 1971; Urry *et al.*, 1971), no direct membrane conformational data support these hypotheses. The gramicidin isolated species 1, 2, and 4 demonstrate the potential of gramicidin to form helical aggregates of either handedness.

Urry *et al.* (1971) reported the synthesis of a covalent dimer in which the two N-termini of desformylgramicidin were linked symmetrically with a malonic acid moiety. This derivative was also reported to be highly active on artificial lipid bilayer membranes. None of the antiparallel-β double-helical conformations postulated here for isolated species 3 could accommodate such a modification, since the formyl groups are at opposite ends of the molecule. Both the parallel-β double helices and the head-to-head π(LD) helical dimers could, in principle, accommodate such a modification.

The reduced artificial lipid bilayer activity (1%) of *N*-acetyldesformylgramicidin reported by Goodall (1971) contrasts with the unaltered equilibrium stabilities of each of the isolated species of this derivative in ethanol. It is possible that the conformational changes due to the substitution of a methyl group for the formyl proton are energetically less costly in solution than in the membrane. Alternatively, the structure of the transmembrane channel might be significantly different from any of the solution structures.

If all of the gramicidin dimers could be demonstrated to be conducting channels, then spectroscopic measurements of the conformation of gramicidin in a suitable lipid bilayer membrane system would yield direct information on the conformation of the gramicidin transmembrane channel, particularly in relation to the conformations of the isolated species.

Summary

(1) The aggregation of gramicidin in nonpolar solvents appears to be a dimerization, as deduced from the concentration profiles and from the concentration dependence of the half-time of forward aggregation. It is likely that none of the gramicidin isolated species are monomers, and, in fact, all may be dimers. Even species 1 and 2, which have very similar conformations, may have the same molecularity.

(2) The dimerization constant is much higher in relatively nonpolar solvents, such as ethyl acetate and dioxane, than in more polar solvents, such as dimethyl sulfoxide, suggesting dimers stabilized by a substantial number of hydrogen bonds.

(3) The very slow aggregation rates for the isolated species in nonpolar solvents suggest structures containing many intermolecular hydrogen bonds, such as the parallel and antiparallel-β double helices.

(4) The relative proportions of the gramicidin isolated species at equilibrium were estimated consistently by both thin-layer chromatography and CD ellipticities.

(5) The gramicidin analog, *N*-acetyldesformylgramicidin, has almost identical dimerization constant and species mole fractions as gramicidin in ethanol. These findings imply that *none* of the isolated species are energetically destabilized by the substitution of a methyl group for the formyl proton.

Appendix

In this appendix the equilibrium and kinetic behavior of an idealized homogeneous monomer-dimer system are developed. When appropriate, the generalization to a conformationally heterogeneous system of monomers and dimers is indicated.

Let $[G_1]$ denote the free monomer concentration (M); $[G_2]$ the dimer concentration (M). The forward rate is k_2^+ (M^{-1} sec^{-1}), and the decay rate is k_2^- (sec^{-1}). In general

$$2G_1 \underset{k_2^-}{\overset{k_2^+}{\rightleftharpoons}} G_2$$

$$\frac{d[G_2]}{dt} = +k_2^+[G_1]^2 - k_2^-[G_2] = -\frac{1}{2}\frac{d[G_1]}{dt} \qquad (1)$$

At Equilibrium. The concentrations are not time dependent, so

$$[G_2]/[G_1]^2 = k_2^+/k_2^- = K_2 \qquad (2)$$

where K_2 is the dimerization equilibrium constant (M^{-1}). Let us define $[G_0]$, the total monomer concentration, as

$$[G_0] = [G_1] + 2[G_2] \qquad (3)$$

Substituting for $[G_2]$ from (2) into (3)

$$[G_0] = [G_1] + 2K_2[G_1]^2 \qquad (4)$$

Dividing through by $[G_0]$ and rearranging, one obtains

$$1 - ([G_1]/[G_0]) = 2K_2[G_0]([G_1]/[G_0])^2 \qquad (5)$$

The mole fraction of gramicidin in monomer is $([G_1]/[G_0])$ and the mole fraction of gramicidin in dimer is $[1 - ([G_1]/[G_0])]$.

Equation 5 still applies to a conformationally heterogeneous system of n dimers, with

$$K_2 = \sum_{i=1}^{n} K_{2_i}$$

where K_{2_i} is the effective dimerization constant for the ith dimer. The fraction of the total dimer in the ith dimer, X_i, is equal to K_{2_i}/K_2.

In the absence of equilibrium two special initial conditions will be considered; the decay experiment where the material is initially dimer and the total concentration is low, and the forward dimerization experiment where the material is initially monomer and the concentration is high.

Low Concentration Decay Experiment. For this case, the forward aggregation term of eq 1 can be neglected, leaving

$$d[G_2]/dt = -k_2^-[G_2] \qquad (6)$$

Integrating and setting $[G_2] = [G_0]/2$ at $t = 0$

$$[G_2] = \frac{[G_0]}{2} e^{-k_2^- t}, \qquad [G_0] << 1/K_2 \qquad (7)$$

This is a simple exponential decay, and the half-time will be equal to $\ln 2/k_2^-$. This experimental decay is independent of conformational heterogeneity or whether the aggregate is dimer, trimer, etc.

High-Concentration Dimerization Experiment. For this case, we may neglect the decay term of (1) and obtain

$$d[G_1]/dt = -2k_2^+[G_0]^2, \qquad [G_0] >> 1/K_2 \qquad (8)$$

Integrating and setting $[G_1] = [G_0]$ at $t = 0$

$$\frac{1}{[G_1]} - \frac{1}{[G_0]} = 2k_2^+ t, \qquad [G_0] >> 1/K_2 \qquad (9)$$

Alternatively, the monomer mole fraction $([G_1]/[G_0])$ can be introduced

$$([G_1]/[G_0])^{-1} - 1 = 2k_2^+[G_0]t, \qquad [G_0] >> 1/K_2 \qquad (10)$$

For a conformationally heterogeneous system of dimers, the same equation will apply with k_2^+ replaced by a weighted sum of the forward rate constants for the individual dimerizations.

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References

Bamberg, E., and Läuger, P. (1973), *J. Membrane Biol. 11,* 177.

Fossel, E.T., Veatch, W. R., Ovchinnikov, Y. A., and Blout, E. E. (1974), *Biochemistry, 13,* 5264

Goodall, M. C. (1971), *Arch. Biochem. Biophys. 147,* 129.

Hladky, S. B., and Haydon, D. A. (1972), *Biochim. Biophys. Acta 274,* 294.

Isbell, B. E., Rice-Evans, C., and Beaven, G. H. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 25,* 192.

Krasne, S., Eisenman, G., and Szabo, G. (1971), *Science 174,* 412.

Myers, V. B., and Haydon, D. A. (1972), *Biochim. Biophys. Acta 274,* 313.

Sarges, R., and Witkop, B. (1965), *J. Amer. Chem. Soc. 87,* 2011.

Urry, D. W. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 672.

Urry, D. W., Goodall, M. C., Glickson, J. D., and Mayers, D. F. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 1907.

Veatch, W. R. (1973), Gramicidin A—Conformations and Aggregation, Ph.D. Thesis, Harvard University.

Veatch, W. R., Fossel, E. T., and Blout, E. R. (1974), *Biochemistry, 13,* 5249.

A ^{13}C Nuclear Magnetic Resonance Study of Gramicidin A in Monomer and Dimer Forms†

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ABSTRACT: Carbon-13 chemical shifts and spin–lattice relaxation times (T_1) have been determined for the random coil and helical states of the linear pentadecapeptide, gramicidin A. Assignments of the resonances were accomplished with the aid of model compounds and gramicidin A analogs from which various amino acids were deleted. The T_1 measurements show that, in DMSO-d_6, the gramicidin A peptide backbone undergoes slowest motion near the center of the molecule (τ_R = 5.0 nsec), while the N-terminal residue moves more rapidly (τ_R = 0.7 nsec). In methanol-d_4, a solvent in which it has been shown that gramicidin A exists predominantly as helical dimer (Veatch *et al.*, 1974, Veatch and Blout, 1974), the T_1 measurements show that motion in the backbone of the molecule has been greatly reduced (τ_R = 30 nsec). This extreme decrease in motion in the peptide backbone going from random coil to dimer form is consistent with the proposed (Veatch *et al.*, 1974) double helical dimer model.

Recently, the first examples of ^{13}C nuclear magnetic resonance (nmr) studies of complex peptides and proteins have been reported. Potentially, chemical shift data, carbon–hydrogen coupling data, and relaxation data should all contribute useful information relating to the structure of the molecules studied. Chemical shift data should be sensitive to local chemical and electronic environmental factors. Although there is information on the chemical shifts of peptides and proteins in the literature (Voelter *et al.*, 1971; Deslauriers *et al.*, 1972; Glushko *et al.*, 1972; Gurd *et al.*, 1972; Jung *et al.*, 1972; Zimmer *et al.*, 1972; Dorman and Bovey, 1973), with minor exceptions it remains largely uninterpretable in terms of specific conformational features. Coupling data potentially contain information regarding angular relationships between atoms. The necessary calibration of coupling constants with bond angles is now beginning to appear (Lemieux *et al.*, 1972). Successful application of relaxation phenomenon to illuminate structural features, such as flexibility and segmental motion of molecules, is becoming more commonplace (Allerhand *et al.*, 1971a,b; Glushko *et al.*, 1972; Komorski and Allerhand, 1972; Levine *et al.*, 1972; Torchia and Piez, 1973; Allerhand and Oldfield, 1973; Brewer *et al.*, 1973; Chiên and Wise, 1973; Torchia and Lyerla, 1974).

The linear peptide antibiotic, gramicidin A, posed structural questions that ^{13}C nmr could help to answer. The amino acid sequence of gramicidin A is given in Table II. All of the amino acid residues are hydrophobic. Although the peptide contains 15 amino acids, it contains only five chemically different amino acids. In addition, if Gly can be designated as a potential D residue, the amino acids in the sequence alternate between L and D configuration. The two preceding papers (Veatch *et al.*, 1974, Veatch and Blout, 1974) have demonstrated that, at high concentration in nonpolar solvents, gramicidin A exists as a family of inter-

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