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# Solution Dimensions of the Gramicidin Dimer by Dynamic Light Scattering†

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ABSTRACT: Gramicidin is thought to form dimeric helical rods in alcohol solutions. In addition, there is evidence that the rod dimensions change upon addition of potassium ions. The present work reports values for the translational and rotational diffusion coefficients of gramicidin in methanol and 95% ethanol and in these same solvents with added KSCN. Solution dimensions are calculated from the diffusion coefficients. The results suggest that gramicidin exists primarily as dimers in these solutions and that the gramicidin rod does indeed become shorter upon addition of potassium ion. These results are consistent with those obtained from X-ray studies on single crystals grown from alcohol solutions.

Gramicidin is a linear pentadecapeptide antibiotic from the bacterium *Bacillus brevis* with alternating L and D amino acid residues. Gramicidin facilitates the diffusion of monovalent cations across membranes by forming NH-terminal to NH-terminal helical dimers (Weinstein et al., 1979) which span the membrane and have a 5–7 Å diameter channel down the center of the helix, through which the monovalent cations can flow. Anions do not flow through these channels whereas divalent cations block the channel and hence prevent further diffusion of monovalent cations through the membranes (Weinstein et al., 1979; Haydon & Hladky, 1972; Veatch & Stryer, 1977; Urry, 1971; Bamberg et al., 1978). X-ray diffraction studies on gramicidin A grown from methanol and ethanol solutions show helical rods with repeat units of 32 Å with 14–16-Å diameters and channels of 4.5–5-Å diameter. Similar studies on gramicidin A complexed to cesium or potassium ions show the helical rods to have repeat units of only 26 Å with 16-Å diameters and 6.8 Å diameter channels (Koeppe et al., 1978, 1979). Fossel et al. (1974) used $^{13}C$ NMR spectroscopy to determine the rotational relaxation time for gramicidin in methanol, for which they obtained $\tau_R = 25$ ns. This is much too slow for a dimer of the dimensions given above.

The present study utilizing dynamic light scattering (DLS) was undertaken to determine whether or not gramicidin exists primarily in dimeric form in solution and, if so, to determine

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the dimer diffusion coefficients and solution dimensions. Dynamic light scattering is particularly well suited for this since both the rotational and translational diffusion coefficients can be determined on the same sample and at sample concentrations that are usually lower than those for $^{13}C$ NMR experiments.

## Experimental Procedures

Gramicidin obtained from ICN Pharmaceuticals as a mixture of gramicidin A (80%), gramicidin B (6%), and gramicidin C (14%) (Weinstein et al., 1979) was dissolved in spectral grade methanol and in 95% ethanol. The solutions were filtered through 0.2-$\mu$m Nucleopore polycarbonate membrane filters into 1-cm$^2$ cuvettes. The samples were determined to be dust free by observing the scattered light through a microscope while 0.4 W of 488-nm argon ion light was shined through the sample.

Translational diffusion coefficients were measured by polarized DLS. A 488-nm vertically polarized argon ion laser beam was focused into a temperature-controlled sample (20.0 ± 0.1 °C for the methanol solutions and 25.0 ± 0.1 °C for the ethanol solutions). The light scattered into angle $\theta$ was focused onto a pinhole. The scattering angle could be varied from 5 to 115°. The light passing through the pinhole was refocused onto the photocathode of an ITT FW-130 photomultiplier tube, and a Mech-Tronics 511 amplifier–discriminator detected the photopulses. The autocorrelation function of these pulses was then calculated by a Malvern 48 channel correlator. Finally, a single exponential fit to the correlation function was performed by a Data General Nova 3 computer by the least-squares method. Typically, ten measurements of the correlation function were taken at each of three scattering angles.

Rotational diffusion coefficients were determined by depolarized DLS. A single-frequency vertically polarized 488-nm argon ion laser beam was focused into a temperature-controlled sample (20.0 ± 0.1 °C for the methanol solutions and 25.0 ± 0.1 °C for the ethanol solutions). The horizontally polarized scattered light was analyzed by a Glan-Thompson polarizer with an extinction ratio of $5 \times 10^{-6}$; fluorescence was removed by a 10 nm bandwidth interference filter centered at 488 nm. The scattered light was then focused onto a 500-$\mu$m pinhole and refocused into a scanning 10-cm confocal Fabry-Perot interferometer. The spectrum was detected by a cooled EMI 9558 photomultiplier tube and a Princeton Applied Research photon counting system. Typically, ten spectra were taken for each sample and fit to a single Lorentzian function by a Data General Eclipse 840 computer.

Potassium thiocyanate was added to one sample in methanol and one in 95% ethanol. Only a limited number of autocorrelation functions and depolarized spectra could be obtained for these samples since aggregation seemed to be initiated by the laser light. When this occurred, data collection was terminated. In the absence of the salt, this aggregation did not occur.

## Results

The translational diffusion coefficient $D$ is determined from the homodyne intensity autocorrelation function $A(I)$ of the polarized scattered light as given in eq 1 (Berne & Pecora, 1976)

$$A(I) = B + Ce^{-2q^2Dt} \quad (1)$$

where $B$ and $C$ are constants, $t$ is time, and $q$ is the length of the scattering wave vector [given by $q = (4\pi n/\lambda) \sin(\theta/2)$,

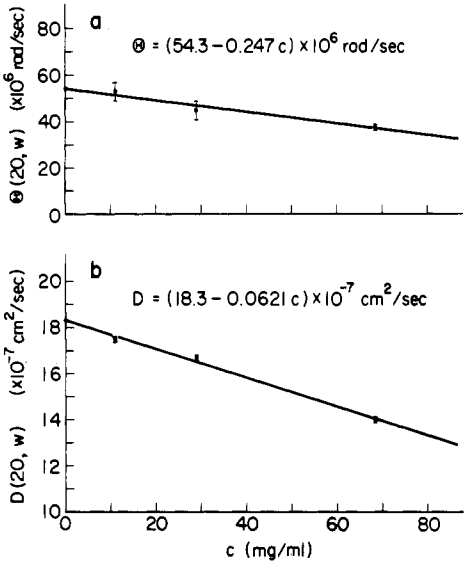


FIGURE 1: Translational (a) and rotational (b) diffusion coefficients of gramicidin in methanol. All values are corrected to a viscosity of 1 cP. Error bars are ±1 standard deviation.

where $n$ is the refractive index of the solution, $\lambda$ is the wavelength of incident light, and $\theta$ is the scattering angle].

The rotational diffusion coefficient $\Theta$ is determined from the spectrum $F(\omega)$ of the depolarized scattered light as given by eq 2 (Berne & Pecora, 1976)

$$F(\omega) = A + B[6\Theta/[\omega^2 + (6\Theta)^2]] \quad (2)$$

where $A$ and $B$ are constants and $\omega$ is the frequency difference between the incident and scattered light beams.

In Table I we present the translational and rotational diffusion coefficients of gramicidin for three concentrations in methanol and 95% ethanol and at one concentration in methanol and 95% ethanol to which 80 mg/mL KSCN was added. The values given for $D$ and $\Theta$ shown are corrected to the viscosity of water at 20 °C in the following way:

$$D(20,\omega) = D(T,s)[\eta(T,s)/\eta(20\ °C,\omega)] \quad (3a)$$

$$\Theta(20,\omega) = \Theta(T,s)[\eta(T,s)/\eta(20\ °C,\omega)] \quad (3b)$$

In eq 3a and 3b $D(20,\omega)$ and $\Theta(20,\omega)$ are the corrected diffusion coefficients and $D(T,s)$ and $\Theta(T,s)$ are the diffusion coefficients determined from eq 1 and 2, respectively, at temperature $T$ and solvent s. $\eta(T,s)$ is the viscosity of the solvent s at temperature $T$ and $\eta(20\ °C,\omega) = 1$ cP is the viscosity of water at 20 °C. Figures 1 and 2 show plots of the corrected diffusion coefficients vs. concentration for the methanol and 95% ethanol solution in the absence of KSCN. The diffusion coefficients obtained by extrapolating to zero concentration are given in Table I.

We obtain solution dimensions for the equivalent ellipsoid of revolution (i.e., the ellipsoid of the revolution with the same diffusion coefficients) from the translational and rotational diffusion coefficients from Perrin's relations for prolate ellipsoids of revolution (Berne & Pecora, 1976)

$$G(\rho) = [6\pi a/(k_BT)]\eta D \quad (4a)$$

$$F(\rho) = [16\pi a^3/(3k_BT)]\eta\Theta \quad (4b)$$

where

$$G(\rho) = \ln[[1 + (1 - \rho^2)^{1/2}]/\rho]/(1 - \rho^2)^{1/2} \quad (5a)$$

$$F(\rho) = [(2 - \rho^2)G(\rho) - 1]/(1 - \rho^4) \quad (5b)$$

Table I: Diffusion Coefficients for Gramicidin in Alcohol Solutions

| solvent | concn (mg/mL) | $D(20,\omega)^a$ ($10^{-7}$ cm²/s) | $\Theta(20,\omega)^a$ ($10^6$ rad/s) |
|---|---|---|---|
| MeOH | 11.0 | 17.5 ± 0.1 | 53 ± 5 |
| | 29.0 | 16.7 ± 0.1 | 45 ± 5 |
| | 68.5 | 14.0 ± 0.1 | 38 ± 4 |
| | 0[b] | 18.3[b] ± 0.1 | 54[b] ± 5 |
| 95% EtOH | 4.0 | 16.3 ± 0.3 | 51 ± 5 |
| | 7.5 | 16.1 ± 0.3 | 52 ± 5 |
| | 8.5 | 16.0 ± 0.4 | 53 ± 5 |
| | 0[b] | 16.6[b] ± 0.4 | 49[b] ± 5 |
| MeOH + 80 mg/mL KSCN | 32.0 | 19.6 ± 0.2 | 73 ± 8 |
| 95% EtOH + 79 mg/mL KSCN | 9.0 | 18.8 ± 0.7 | 73 ± 8 |

[a] Corrected to 1 cP; errors given are ±1 standard deviation. [b] Extrapolated to zero concentration.

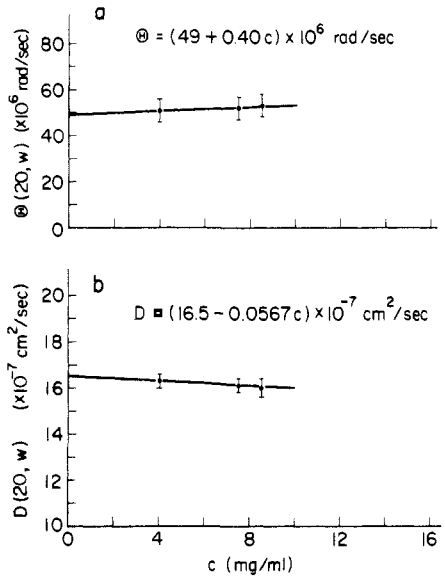


FIGURE 2: Translational (a) and rotational (b) diffusion coefficients of gramicidin in 95% ethanol. All values are corrected to a viscosity of 1 cP. Error bars are ±1 standard deviation.

and $\rho = b/a$; $a$ and $b$ are the lengths of the semimajor axis and semiminor axis, respectively, $k_B$ is Boltzmann's constant, and $T$ is the absolute temperature. The lengths of the semimajor and semiminor axes $a$ and $b$ obtained from eq 4 and 5 are given in Table II. Although there are no equations similar to Perrin's relations for relating the diffusion coefficients to the dimensions of a short rodlike molecule, we can obtain approximate dimensions by finding a rod with the same volume as that of the ellipsoid of revolution (Tanford, 1961). Unfortunately, there are an infinite number of such rods; thus, a further approximation is required. We assume, therefore, that the radius of the rod is equal to the length of the semiminor axis of the ellipsoid of revolution. The dimensions of the rod obtained by us are given in Table II along with those of the crystal obtained in the X-ray studies of Koeppe et al. (1978, 1979).

## Discussion

The agreement between our results and those of Koeppe et al. is excellent, as shown in Table II. Although the errors in the derived rod dimensions are rather large due to the 10% error in the rotational diffusion coefficients, the large change in both the rotational and translational diffusion coefficients on adding KSCN demonstrates that there is a significant change in dimensions of the diffusing particles. This change

Table II: Dimensions of Equivalent Ellipsoid, Diffusing Rod, and Crystal

| | prolate ellipsoid dimensions | | rod dimensions | | X-ray | |
|---|---|---|---|---|---|---|
| solvent | semimajor axis (Å) | semiminor axis (Å) | length (Å) | diam (Å) | length (Å) | diam (Å) |
| MeOH | 23 ± 2 | 6.8 ± 0.7 | 31 ± 3 | 14 ± 2 | 32 | 15 |
| EtOH | 22 ± 2 | 8.4 ± 0.9 | 29 ± 3 | 17 ± 2 | 32 | 15 |
| MeOH + KSCN | 20 ± 2 | 7.7 ± 0.7 | 27 ± 3 | 15 ± 2 | 26 | 16 |
| EtOH + KSCN | 19 ± 2 | 8.6 ± 0.8 | 25 ± 3 | 17 ± 2 | | |

in diffusion coefficients on adding KSCN could be due to some of the gramicidin dimers dissociating into monomers. A mixture of monomers and dimers would result in an apparent reduction in the rod dimensions as observed; we cannot distinguish this from a change in dimer structure on adding potassium ions as observed by Koeppe et al. Nevertheless, the excellent agreement between the dimensions obtained by DLS in this work and those obtained from X-ray crystallography by Koeppe et al. for gramicidin dimers suggests that we are observing the same structural changes on adding potassium ions.

Fossel et al. (1974) determined the rotational time of gramicidin in methanol at 100 mg/mL by $^{13}C$ NMR, for which they obtained $\tau_R = 25$ ns. We can obtain the rotational diffusion coefficient for gramicidin in methanol at 100 mg/mL by use of Figure 1a. The least-squares line through the data is

$$\Theta = (54.3 - 0.247c) \times 10^6 \text{ rad/s}$$

where $c$ is in milligrams per milliliter. At 100 mg/mL gramicidin in methanol, $\Theta = 30 \times 10^6$ rad/s. Since the rotational time $\tau_R$ measured in a $^{13}C$ NMR measurement is related to $\Theta$ by

$$\tau_R = 1/(6\Theta)$$

we find $\tau_R = 5.6$ ns. Fossel et al. state that their rotational time is much too slow for a rod of the dimensions expected for gramicidin dimers. Indeed, our results show that their rotational time is a factor of 4 too slow. In $^{13}C$ NMR measurements of spin relaxation the $T_1$ relaxation times give two possible rotational times [see Fossel et al. (1974)]. They used $T_2$ relaxation times and the nuclear Overhauser effect (NOE) to determine which value of $T_1$ gave the correct value of the rotation time. We can only suggest that they chose the wrong value of $T_1$ in their analysis. This demonstrates the advantage of measuring diffusion coefficients by DLS where relatively low concentrations can often be used and the measurements determine overall molecular motions rather than those of individual carbon atoms. Thus, the interpretation of the light scattering results is often considerably simpler.

In conclusion, we believe gramicidin exists primarily as dimers in ethanol and methanol solutions with dimensions consistent with those found in crystals grown from these solvents. Likewise, the gramicidin dimers undergo structural changes that appear to be the same as those observed in crystals when potassium ions are introduced.

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# Chemical Cleavage of Tryptophanyl and Tyrosyl Peptide Bonds via Oxidative Halogenation Mediated by *o*-Iodosobenzoic Acid†

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ABSTRACT: The procedure to cleave tryptophanyl peptide bonds in proteins using *o*-iodosobenzoic acid (IBA) [Mahoney, W. C., & Hermodson, M. A. (1979) *Biochemistry 18*, 3810–3814] has been reexamined in order to clarify the mechanism of the cleavage reaction. It was found that IBA, under the proposed experimental conditions of protein fragmentation (80% aqueous acetic acid/4 M guanidine hydrochloride), mediates the oxidative chlorination of the indole nucleus of tryptophan and of the phenol ring of tyrosine. The mechanisms of the peptide bond fissions are thought to be similar to those already proposed for other reagents of the positive halogen type (e.g., *N*-bromosuccinimide). Several tryptophanyl and tyrosyl peptides were cleaved in 24–83% yields. It was shown that halides are a necessary reactant for the IBA-mediated peptide bond fission to occur. Excess *p*-cresol added to the reaction mixture as a scavenger for tyrosine modification allows selective cleavage at tryptophan. Horse heart cytochrome *c*, which contains one tryptophan residue and four tyrosine residues, was not cleaved by IBA alone but extensively cleaved at both these amino acids when the reaction was carried out in the presence of 4 M guanidine hydrochloride. On the other hand, in the presence of *p*-cresol, cleavage at tryptophan occurred quite selectively, and protein fragments 1–59 and 60–104 were isolated after gel filtration in ~60% yield. It is concluded that incubation of IBA under acidic conditions and in the presence of halides involves a series of equilibrium reactions leading to the formation of halogenating species, including free halogen. In particular, modification and cleavage at tyrosine are not due to *o*-iodoxybenzoic acid, thought to be present in the commercially available IBA reagent [Mahoney, W. C., Smith, P. K., & Hermodson, M. A. (1981) *Biochemistry 20*, 443–448], but is an inherent property of the IBA/guanidine hydrochloride/acetic acid reagent. In fact, the purity of the IBA sample used throughout this study was determined by numerous criteria, including elemental analysis, iodometry, and thin-layer as well as column chromatography.

Several procedures have been explored in the past to cleave tryptophanyl peptide bonds in proteins (Witkop, 1961; Spande et al., 1970; Fontana & Toniolo, 1976; Fontana et al., 1980). Most of the reagents employed were of the positive halogen type, such as *N*-bromosuccinimide (NBS)[1] (Patchornik et al., 1960; Ramachandran & Witkop, 1967), 2,4,6-tribromo-4-methylcyclohexadienone (tribromocresol) (Burstein & Patchornik, 1972), *N*-chlorosuccinimide (Shechter et al., 1976; Lischwe & Sung, 1977), 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine (BNPS-skatole) (Omenn et al., 1970; Fontana, 1972; Fontana et al., 1973), and dimethyl sulfoxide ($Me_2SO$) in the presence of hydrobromic acid (Savige & Fontana, 1977c; Fontana et al., 1980). Presently, the most often used method for cleavage at tryptophan utilizes BNPS-skatole as a source of positive halogen. This reagent is highly selective, and yields of cleavage are satisfactory (up to 80%) [see Fontana et al. (1980) for references].

Recently, *o*-iodosobenzoic acid (IBA) has been reported to cleave specifically tryptophanyl peptide bonds in proteins with 70–100% yields (Mahoney & Hermodson, 1979). However, subsequent reports (Fontana et al., 1980; Wachter & Werhahn, 1980; Johnson & Stockmal, 1980) showed that the reagent was not as selective as orginally reported since peptide bond cleavage at tyrosine was demonstrated to occur in moderate to high yields when the procedure was applied to several proteins (cytochrome *c*, myoglobin, and actin). More recently (Mahoney & Hermodson, 1980; Mahoney et al., 1981), it was proposed that *o*-iodoxybenzoic acid, thought to be present as a contaminant in the commercially available sample of IBA, is responsible for the observed cleavage at tyrosine.

The results of the studies reported herein rule out the role of contaminants in the IBA-mediated peptide bond fissions and show that the reagent, under the proposed experimental conditions for protein fragmentation (80% aqueous acetic acid/4 M Gdn·HCl), mediates the oxidative chlorination of

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[1] Abbreviations used: IBA, *o*-iodosobenzoic acid; BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; NBS, *N*-bromosuccinimide; $Me_2SO$, dimethyl sulfoxide; Oia, oxindolylalanine (2-hydroxytryptophan); Dia, dioxindolylalanine; Gdn·HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane; RNase, bovine pancreatic ribonuclease A (amino acids and amino acid derivatives have the L configuration).

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