LIGHT-SCATTERING STUDIES OF THE MONOMER-DIMER STATES OF GRAMICIDIN A

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ABSTRACT Measurements of the intensity and autocorrelation function of light quasielastically scattered by gramicidin A indicate that the molecules exist as dimers in methanol and dioxane and monomers in dimethyl sulfoxide when solute concentrations range from 18 to 50 mg/ml. This gives further evidence that gramicidin A can dimerize in low-polarity media such as the hydrocarbon part of the membrane bilayers.

INTRODUCTION

Early experiments by Sarges and Witkop (1) on the membrane-active peptide antibiotic gramicidin A (GmA) have given evidence of association to dimers in solvents with low dielectric constant (e.g., dioxane). This is clearly of great importance in understanding GmA-induced ionic conductivity in natural (2) or artificial (3) membranes. In the generally accepted mode (4, 5) the conducting structure consists of at least two polypeptide molecules spanning the lipid bilayer and forming a pore that passes univalent cations through the membrane. Thus it is important to know the conditions under which GmA readily dimerizes.

We wish to report the results of a light-scattering study of the molecular weight and diffusion constant of GmA in three solvents of increasing polarity: dioxane, methanol, and dimethyl sulfoxide (DMSO). Both measurements are consistent with a configuration predominantly dimer in dioxane and methanol and monomer in DMSO at the concentrations studied (~30 mg/ml). The solvated dimer is roughly cylindrical with a diameter of ~2.6 nm and length 5.0 nm. The results confirm work of Veatch and Blout (6) by an entirely different method and contribute to a coherent picture of the monomer-dimer association properties of GmA in different solvents.

EXPERIMENTAL PROCEDURE

Commercially available gramicidin was used as received from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp. (Cleveland, Ohio). Actually, this product is a mixture (7) of 72% GmA, 9% GmB, and 10% GmC, but since these three gramicidins differ by only one amino acid, we believe our results are representative of GmA. All solvents used were analytical reagent grade. To ensure equilibrium, methanol and DMSO solutions were allowed to equilibrate for 24 h. The solubility of GmA in dioxane was increased by adding ~0.5% HCl and the solution was allowed to equilibrate for 1 wk. Just

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before use, the solutions were centrifuged at 6,400 *g* for 1 h and then introduced into the scattering cell through two successive 0.22-μm Millipore Teflon filters (Millipore Corp., Bedford, Mass.). The cell had a volume of 0.3 ml and was constructed so the solution came into contact with only glass and teflon. No change in GmA properties in the cell was observed over periods of as long as 1 wk. Extreme cleanliness of the cell is necessary; this was achieved by using a procedure described by Dubin (8). The temperature of the cell was controlled to 20 ± 0.1°C with an electrical oven equipped with a thermoelectric Peltier element.

Diffusion constants were measured by light-beating spectroscopy (9, 10). The technique is well described in the literature, although our work is the first reported with molecules as small as GmA, whose mol wt, M_w is ~2,000.

In a light-beating experiment the signal-to-noise ratio is proportional to the number of scattered photons per correlation time in a coherence area, and also increases proportionally to the square root of the observation time (9). The number of scattered photons per correlation time in a coherence area is proportional to $M_w c\,(dn/dc)^2/Dq^2$ for molecules of mol wt M_w, diffusion constant D, and concentration c in a solution whose refractive index is n (11). Here $q = (4\pi n/\lambda) \sin \Theta/2$ is the scattering wave vector for a scattering angle Θ. For molecules of similar shape, D should vary roughly as $M_w^{-1/3}$. We used an argon laser (Spectra-Physics model 165, λ = 5,145 Å, Spectra-Physics Inc., Mountain View, Calif.) with a power output of 200 mw and a real-time digital autocorrelator (Precision-Devices, Malvern type K 7023, Precision Digital Corp., Needham, Mass.) to analyse the scattered light. Laser powers much larger than 200 mw resulted in convection currents and Doppler shifts due to local heating of the solution. Occasionally, the experiments were conducted with smaller laser outputs ~50 mw to check possible disturbances in the sample by the laser beam. The digital correlator provided both stability for the long observation times, typically 15–30 min, and short-time resolution (~100 ns) necessary with the large Doppler shift of small molecules, as well as a precise determination of the correlation function. With little difficulty we were able to study GmA (M_w = 2,000) at Θ = 90° with $c > 20$ mg/ml. To extend this to somewhat smaller molecules at the same concentration, or to lower concentration of GmA becomes more difficult, as the signal due to the solute molecules is hardly bigger than that due to the solvent.

The molecular weight measurements were carried out by the usual method of measuring the intensity of polarized light scattered at 90° (12); however, digital counting techniques were used to improve stability and accuracy. The apparatus is a modification of that designed by one of us to measure the sizes of particles <10 nm in size (13). A photomultiplier (ITT FW 130, ITT Electro-Optical Products, Fort Wayne, Ind.) was alternatively illuminated (at 240 Hz) with light scattered by GmA solution or with an attenuated beam from the illuminating laser; the photoelectrons for the two beams were directed to the two inputs of a digital counter operating in the ratio mode. This system had excellent stability and enabled us to determine reliably the ratio of scattered light to incident light with an accuracy better than 0.5%.

The system was calibrated by using an aqueous solution of lysozyme (M_w = 14,500, dn/dc = 0.188 ml/g) (14) at pH 1.6, whose concentration was adjusted so the scatterred intensity was about the same as the GmA solution. For GmA solutions we measured dn/dc to be 0.210 ± 0.06 in methanol and 0.0745 in DMSO at 23°C. The difference between the two values arises from the different refractive indices of the solvents. These results are in good agreement with the equation $dn/dc = \phi\,(n_p - n_s)$ taking a partial specific volume ϕ = 0.83 ml/g and n_p = 1.600 for the protein (15). The concentrations in the solutions were determined by measurements of the optical absorption due to the tryptophan residues of the GmA molecule. An independent calibration gave extinction coefficient values for a 1-cm path at 1% concentration of E = 108 ± 1 at 280 nm in methanol and 107 ± 1 at 183 nm in DMSO.

RESULTS

The results of measurements at 23°C are given in Table I for methanol and DMSO solvents. We estimate experimental accuracy to be 5% at c = 30 and 50 mg/ml in methanol, but only 10% at c = 18 mg/ml in methanol or at any concentration in DMSO, since in the latter

TABLE I
MOLECULAR WEIGHT OF GmA

Solvent	c = 18 mg/ml	c = 30 mg/ml	c = 50 mg/ml
		mol wt	
Methanol	4,150	4,100	3,925
DMSO	—	2,100	2,300

situations the scattering from GmA is barely larger than the Rayleigh scattering from the solvent. No corrections were made for concentration dependence since the second virial coefficient B is sufficiently small, $\simeq 3 \times 10^{-5}$ for monomer gramicidin in dimethylformamide (1), so the correcting term $2Bc$ is small compared to $1/M$.

We may compare our results with previous information about the aggregation of GmA. Our light-scattering measurements show that GmA, whose chemical mol wt is 1,880, at concentrations around 30 mg/ml, is a dimer in methanol and dioxane and a monomer in DMSO. On the other hand, Sarges and Witkop (1) report dimers in dioxane and monomers in ethanol at concentrations apparently below 10 mg/ml. However, Veatch and Blout (5), by fluorescence polarization studies, show the aggregation of Gm in methanol changes over the range $c \sim 2$ mg/ml (presumably monomers) to 20 mg/ml (dimers); thus all of these results appear to be consistent, and our results show unambiguously that the aggregated state for $c >$ 20 mg/ml is a dimer. In DMSO, Veatch finds only a slight increase in aggregation up to the solubility limit of 200 mg/ml. Our data show a mol wt of 2,100 at 30 mg/ml and an increase to 2,300 at 50 mg/ml. These results are consistent with an essentially monomer state but with ~10 and ~15% dimerization for the two concentrations. They could also be explained by the presence of minute particulate impurities.

The results of our measurements of the diffusion constant of GmA under various conditions are summarized in Table II. The 20-point autocorrelation function for the scattered light was analyzed by the method of cumulants developed by Koppel (16) to determine the average diffusion coefficient $\overline{D}$ and its variance V; the latter provides a measure of the polydispersity of the molecules in solution. Each correlation function was analysed for both two and three cumulants, and data were taken at scattering angles of 30, 60, and 90°; under all these conditions the results were consistent with the values of $\overline{D}$ reported. In particular, a plot of

TABLE II
DIFFUSION CONSTANT OF GmA

Solvent	T	D	V	$\eta^* \times D$
	°C	*$\times 10^7$ cm²/s*	*%*	*$\times 10^9$ dyn*
Methanol	20	19 ± 3	45	11.7 ± 2.4
(50 mg/ml)	25	21 ± 2	50	12.4 ± 1.2
Dioxane	20	8.4 ± 2.0	50	12.9 ± 1.5
(50 mg/ml)	25	10.0 ± 1.0	45	13.1 ± 1.3
DMSO	25	7.0 ± 1.0	60	15.9 ± 2.0
(30 and 50 mg/ml)				

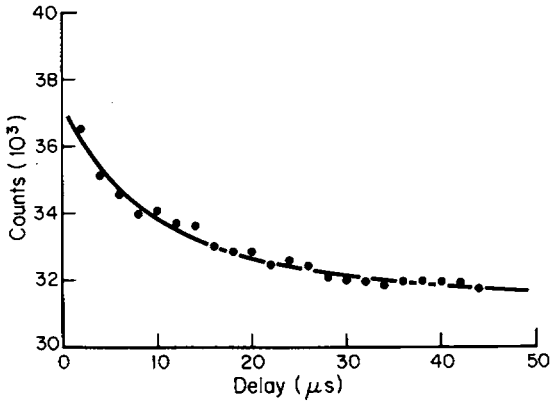

FIGURE 1 The autocorrelation function for light scattered from GmA in methanol ($q = 1.42 \times 10^5$ cm^{-1}); the solid line is the cumulants fit. The data (solid points) were accumulated in about 24 min.

the mean correlation time $\bar{\Gamma}$ vs. q^2 goes through the origin as expected for a purely diffusive process. The values of $\bar{D}$ given in Table II were obtained in each case from the slope of these curves. There were no systematic differences in the determination of $\bar{\Gamma}$ and of its variance between the 2 and 3 cumulants fits. Data values were scattered typically within 10%.

A typical autocorrelation function and the resulting cumulants fit are shown in Fig. 1. The experimental baseline at infinite times is in good agreement with the baseline calculated from the monitor channels, which further justifies our analysis of the data. The effective viscosity, η^*, used in the table was calculated from the Einstein-Simha equation (11) and differs from that of the pure solvent by ~5%. The product $\eta^*\bar{D}$ should be constant for molecules of the same hydrodynamic radius, and indeed has the same value in dioxane and methanol within the experimental uncertainty. The variance of 50% is rather large compared to that obtained for polystyrene spheres used as calibration standards, and it is tempting to take this as an indication of polydispersity in the sample. However, we calculate that a 50% variance could also be caused by the presence of 100-nm dust particles (which could pass through our 200-nm filter) in the ratio of 1 for each 50,000 GmA molecules. In fact, we observed a significant increase in the variance and of the average counting rate at low scattering angles where the larger particles would scatter more strongly. Thus, we cannot be certain the variance represents a real polydispersity of the GmA. The possibility of explaining the variance by multiple scattering (17) seems excluded here, since we are using very low solute concentrations.

DISCUSSION

We complete our picture for the configuration of the GmA molecule in various solvents by discussing the diffusion constant measurements within the framework of the Perrin model (18) for the diffusion of ellipsoids of resolution. Our experiments measure the time for a molecule to diffuse a wavelength of light, which is at least 1,000 times longer than the reorientation time for rotational Brownian motion; therefore we measure the angle-averaged

translational diffusion constant for the GmA monomers and dimers. This is given by

$$D^T = \frac{kT}{6\pi\eta a} G(b/a),$$

where a and b are the semi-axes. For prolate spheroids ($a > b$)

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

and for oblate spheroids ($a < b$)

$$G(b/a) = [(b/a)^2 - 1]^{-1/2} \arctan [(b/a)^2 - 1]^{1/2}.$$

One may calculate from these expressions that D^T remains within 10% of the value for spheres ($a = b$) as a/b ranges from 0.01 to 6.0 if the surface area of the spheroid is constant. Therefore our measurements of D^T determine the relative hydrodynamic surface areas of the monomer and dimer forms of GmA but do not enable us to make a quantitative statement about the shape of either form. From the data in Table II we calculate the dimer in dioxane has an effective hydrodynamic surface area 1.5 times that of the monomer in DMSO, and the dimer in methanol has an effective area 1.6 times that of the monomer. These ratios are consistent with the end-to-end stacking of monomers with any of the proposed double helical structures (19, 20).

To summarize, we have shown that through digital autocorrelation methods the technique of light-beating spectroscopy may be extended to measure the diffusion constant of molecules with mol wt as low as 2,000. The advantage of the digital correlation method is apparent when we point out that GmA in DMSO would yield a signal-to-noise ratio two orders of magnitude smaller under the same experimental conditions as the smallest molecule previously studied by light-beating spectroscopy (21). Thus conformational changes in a variety of "ionophores" (various chelates and antibiotics such as valinomycin) of low molecular weight are amenable to experimental investigation by light scattering.

Our measurements show that GmA molecules form dimers in methanol and dioxane, and remain monomeric in DMSO when the concentrations range from 18 to 50 mg/ml. Raman spectra (22) suggest the conformation of GmA is different in DMSO than methanol or dioxane; this is presumably related to its monomeric state in DMSO. Our data provide confirmation by a different and more direct technique of Veatch's results. Thus the trend is to form dimers more readily in less polar environments; this gives further support to the hypothesis that gramicidin aggregates serve to facilitate the passage of cations through lipid membranes.

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