Published in final edited form as:

J Phys Chem B. 2011 June 9; 115(22): 7427–7432. doi:10.1021/jp200906y.

Gramicidin A Backbone and Side Chain Dynamics Evaluated by Molecular Dynamics Simulations and Nuclear Magnetic Resonance Experiments. II: Nuclear Magnetic Resonance Experiments

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Abstract

Motional properties are important for understanding protein function and are accessible to NMR relaxation measurements. The goal of this study is to investigate the internal dynamics occurring in gramicidin A (gA) channels, in order to provide benchmark experimental data for comparison with the results of molecular dynamics simulations. We therefore synthesized several ¹⁵N isotope-enriched gA samples, covering all backbone residues as well as the Trp indole side chains for NMR relaxation experiments. Based on the ¹⁵N-NMR spectra for labeled gA samples incorporated in sodium dodecylsulfate (SDS) micelles, we have determined T₁, T₂, and heteronuclear NOE values for backbone and indole ¹⁵NH groups. The results indicate that the SDS-incorporated gA channel is a constrained structure, without an especially “floppy” region. The NMR observables, particularly those for backbone groups, are predicted well by the molecular dynamics simulations in the accompanying article.

Keywords

gramicidin channels; SDS micelles; NMR relaxation

Introduction

Increasingly, progress in membrane biophysics demands mutual cooperation and feedback between theory and experiment. In this and the accompanying article¹, we have undertaken a combined investigation of the folded channels formed by the benchmark gramicidin A (gA), a pentadecapeptide from the soil bacterium *Bacillus brevis*. The gA sequence consists of alternating L- and D- amino acids^{2,3}. This unique pattern allows the gramicidins to fold into a variety of helical conformers^{4–7}, including the right-handed, single-stranded β^{6,3} helical

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structure in the bilayer-spanning channels. In lipid bilayer membranes, the conducting gA channels are head-to-head dimers that are stabilized by hydrogen bonding at the bilayer center and tryptophan indole hydrogen bonds to polar groups at the bilayer/solution interface. The channel dimer forms a central pore with a diameter of ~ 4 Å that confers a specificity for monovalent cations. The pore is lined by amide hydrogens and carbonyl oxygens. Permeating cations therefore interact directly with peptide backbone groups, making it important to investigate the backbone dynamics to obtain insights into the correlation between the internal motion and ion permeation. Within this context, the relatively small size of gA makes it a suitable candidate for combined spectroscopic and molecular dynamics (MD) simulation studies of channel structure, function and dynamics⁸, though the presence of L- and D-amino acid residues raises additional challenges for the computations.

Several previous studies have addressed the global dynamic parameters of gA dimers in lipid environments. Global correlation times in dimyristoylphosphatidylcholine (DMPC) bilayers, estimated from deuterium NMR lineshape analysis, have been reported⁹; local backbone dynamics have been investigated to a smaller extent. Solid-state ^{15}N T_1 relaxation measurements have been performed for several labeled amino acids in the sequence¹⁰. A detailed analysis of a single peptide plane using multiple NMR constraints yielded an order parameter of ~ 0.93 ¹¹.

Here we report the ps-ns scale backbone dynamics^{12,13} of gA incorporated into sodium dodecylsulfate (SDS) micelles. Heteronuclear ^1H - ^{15}N spin-lattice and spin-spin relaxation magnitudes as well as heteronuclear NOE values were obtained for each amino acid linkage in the peptide and analyzed within a model-free formalism. The accompanying article¹ reports the computational predictions of the NMR relaxation and NOE parameters for gA dimers in SDS micelles, as well as potential of mean force calculations for the movement of Na^+ and K^+ through gA channels in DMPC bilayers. We find that the MD simulations predict well the NMR observables for the peptide backbone dynamics. The side chain dynamics are predicted less well—presumably because of the relatively slow side chains motions, which are not sampled adequately in the simulations.

Materials and Methods

Fmoc-protected ^{15}N enriched amino acids were obtained from Cambridge Isotope Laboratories (Andover, MA), except for Trp which was bought without N- α -Fmoc protection. Tryptophan, containing two ^{15}N labels (backbone and side chain) was modified with a Fmoc group, using the procedure described previously¹⁴. The reaction product was recrystallized from ethyl acetate:hexane, 4:1. Successful incorporation of Fmoc moiety was confirmed by ^1H NMR in d_6 -DMSO. A total of four peptides were synthesized, they are listed in Table 1.

Commercial Wang resin from NovaBiochem (San Diego, CA) was used for all the peptides, apart from the one used to synthesize the Trp13 and Trp15 labeled gA. In this case, ^{15}N enriched Fmoc-Trp was manually loaded on the Wang resin, using N, N'-diisopropylcarbodiimide (Sigma-Aldrich, St. Louis, MO) as an activating agent. Peptides were synthesized on Perkin-Elmer Applied Biosystems 433A synthesizer (Foster City, CA), using standard FastMoc chemistry. Upon completion of the synthesis, the N-terminal valine was formylated by reaction with *para*-nitrophenylformate in dimethylformamide for 24 hours. Both resin loading and formylation were done at 4 °C to reduce the risk of racemization. Formylated peptides were released from the resin by ethanolamine cleavage, resulting in the C-terminal ethanolamide group.

Peptides were purified from low molecular weight contaminants (formylation and cleavage cocktail components) by size-exclusion chromatography, using two sequentially connected glass columns (1000×30 mm) packed with LH20 hydrophobic sorbent. Linear flow of 100% methanol at 1.5 ml/min was applied. Detection was done spectrophotometrically at 280 nm. Fractions were collected every 10 minutes and analyzed by HPLC in isocratic conditions (91% methanol, 9% water; each eluent contained 0.1% trifluoroacetic acid). Similar fractions were combined, concentrated *in vacuo* and dried down using vacuum centrifugation (10 mtorr vacuum). Purity and identity of the peptides were confirmed by analytical HPLC and mass spectrometry (Figure 1).

Perdeuterated sodium dodecylsulfate (d_{25} -SDS) was recrystallized from 95% ethanol at -20°C . Gramicidin A (5 μmol) was dissolved in 50 μl of perdeuterated (d_3) trifluoroethanol and added to 350 μmol of d_{25} -SDS in phosphate buffer, pH 7.0 : D_2O , 9 : 1 (0.7 ml total volume). The dispersion was sonicated extensively to promote peptide incorporation into detergent micelles and to break down large SDS aggregates, and then centrifuged at 10,000 rpm using a desktop centrifuge. The supernatant was transferred to an NMR tube, which was stored in the dark to prevent photooxidation of Trp residues.

Longitudinal (T_1) and transverse (T_2) relaxation times and steady-state $15\text{N}\{-^1\text{H}\}$ NOE enhancements were recorded on a 500 MHz Bruker Avance spectrometer at 30, 40 and 55°C utilizing conventional pulse sequences¹². The pulse durations were in the range of 8.3–9.6 μs , with the exact value dependent on the sample and temperature. The recycle delay was 1 s (T_1 , T_2) or 4 s (NOE). The temperature was calibrated using methanol¹⁵. Spectral widths were 12 ppm (^1H) and 70 ppm (^{15}N). Data were acquired in a 4096×256 matrix with 32 (T_1 , NOE) or 64 (T_2) scans per increment, and Fourier transformed into a 8192×2048 set, using quadratic sine weighting functions with a sine bell shift value of 5 in both dimensions. Relaxation measurements included 15–17 delay times, spanning the range of 15 ms to 2 s (T_1) and 10 ms to 0.35 s (T_2). Cross peaks in 1H - 15N spectra were integrated using TopSpin (Bruker) and fitted to a monoexponential decay function, $V = A \cdot \exp(-\tau/T_{1,2})$, where V is the peak volume and τ is the delay time, using a grid search routine. The steady-state NOE values were determined as the ratio of peak volumes from the experiments with and without proton presaturation (4 s).

Relaxation analysis for backbone residues was performed using Relax software^{16,17}. The length of the NH bond was fixed at 1.02 Å and chemical shift anisotropy was set to -160 ppm¹⁸. A spherical diffusion tensor model was employed to describe the global tumbling of the SDS-gramicidin complex. Local bond motions were accounted for using a generalized order parameter^{19,20}.

Results

Previously reported ^1H - ^{15}N spectra showed largely overlapping signals in the backbone region and particularly in the Trp indole region of the spectra, complicating the signal integration and analysis^{21,22}. To improve peak dispersion, we synthesized several different peptides with selective introduction of ^{15}N labels to ensure minimal overlap of resonances in a particular HSQC spectrum. For this reason, the peptides listed in Table 1 were chosen. Rather than using isotope enriched D-amino acids, which are fairly expensive, we labeled the corresponding L-residues in the enantiomeric gA^- , which forms $\beta^{6.3}$ helices (and bilayer-spanning channels) of opposite chirality to gA^{23} —with properties otherwise equivalent to those of native gA channels²⁴. The HSQC spectra of the resulting peptides (Figure 2) exhibit sharp well-resolved resonances, indicating the homogenous environment of the micelles.

Relaxation decay curves were obtained from fitting the peak volumes to a monoexponential decay (Figure 3). Extensive sampling of delay times ensured accurate determination of the relaxation times. Spin-lattice (T_1) relaxation values of the backbone amide groups are similar throughout the backbone (Figure 4a). There is somewhat larger dispersion in the spin-spin (T_2) relaxation results, which is primarily caused by larger errors associated with the experiment (Figure 4b). The similarity of the T_1 and T_2 values for each of the residues throughout a peptide is consistent with a rigid structure of the channel, which parallels observations using solid state NMR¹⁰.

Steady-state heteronuclear NOE values (Figure 4c) are highly consistent throughout the sequence, with an average value of 0.72 (at 55 °C). Such fairly high NOE suggest only minor internal motion, consistent with the earlier reports on selectively labeled gA¹¹.

The tryptophan residues at the lipid bilayer—water interface are important for subunit folding²⁵, channel formation²⁶ and function²⁷. Due to the small pitch in a $\beta^{6,3}$ helix, the polarity of the environment is similar for each of the four Trp residues, as seen in the NMR structures for the gA channel in SDS micelles or in DMPC bilayers^{21,28,29}. The reported geometries of gA are similar, apart from a few minor aspects such as Trp9 side chain orientation that is stacked with Trp15 indole in the structure derived from solid-state NMR, and the solid-state and solution NMR structures can be reconciled through MD simulations⁸. ¹³C solid-state NMR experiments also have been employed to deduce the Trp side chain orientation; they yielded similar results in micelles and DMPC lipid bilayers^{30,31}. Several earlier studies have examined the motion of tryptophan side chains in gA. Libration motions around the χ_2 angle, of approximately $\pm 20^\circ$ amplitude, have been reported for all four tryptophan residues using solid-state lineshape analysis of gA in DMPC under cryogenic conditions²⁸; while solid-state NMR experiments in hydrated DMPC bilayers at elevated temperature suggested somewhat different motions for the different Trp side chains^{32–34}.

To probe the side chain behavior, in addition to the backbone amide dynamics, we also collected relaxation data for the indole $N_\epsilon H$ groups in the Trp side chains. Whereas the T_1 and T_2 values for the four tryptophan residues are similar within the experimental error, there is some variation in the steady-state NOE enhancements. Overall, the NOE values for the side chains are 0.2–0.3 units higher than those for the corresponding backbone signals, indicating the increased side-chain flexibility. The NOEs for Trp9 and Trp11 are comparable, the NOE for Trp13 is somewhat higher, and that for Trp15 is lower. It is of interest that the highest NOE is observed for Trp13, as higher NOE values usually are associated with a decrease in motion (larger order parameter). All four tryptophan side chains are exposed to aqueous environment to some extent, although water accessibilities are not identical³⁵. In this context, the lower NOE of the Trp15 side chain is likely to be due to increased exposure to less viscous and less sterically prohibitive water environment, allowing for more conformational freedom. Similar effects on NMR observables were observed in the proton NMR of the indole side chains, though the changes were more gradual³⁶. It is of interest that the NOE values for both the Trp13 and Trp15 backbone nitrogens are similar to those for the other backbone groups in the peptide. Overall, the relatively high NOE values seem to indicate that all four tryptophan side chains undergo restricted motion, similar to the previous observations using proton relaxation³⁶ and deuterium solid-state NMR³³.

To express the NMR observables in terms of molecular motion, we analyzed the relaxation data within Lipari-Szabo model-free formalism^{19,20} using Relax software^{16,17}. The relaxation of the ¹⁵N nuclei occurs by a dipolar coupling mechanism to a bound proton, with

a minor contribution from chemical shift anisotropy. We utilized the following form of spectral density at frequency ω (Eq. 1):

$$J(\omega) = \frac{2}{5} \left(\frac{S^2 \tau_m}{1 + (\omega \tau_m)^2} + \frac{(1 - S^2) \tau}{1 + (\omega \tau)^2} \right) \text{ with } \tau = \frac{\tau_m \tau_e}{\tau_m + \tau_e}. \quad (1)$$

Here the global motion is defined by the molecular correlation time τ_m , and the local bond dynamics in the ps to ns time scale by a generalized order parameter S^2 and extra correlation time τ_e , under the assumption that the time scales of the global and internal dynamics are significantly different, i.e. $\tau_m \gg \tau_e$ ^{19,20,37}. Under the condition of extreme narrowing regime $((\tau_e \omega)^2 \ll 1)$, the contribution from the second term in Eq. 1 is negligible, and the spectral density can be rewritten as Eq. 2¹³:

$$J(\omega) = \frac{2}{5} \left(\frac{S^2 \tau_m}{1 + (\omega \tau_m)^2} \right). \quad (2)$$

Because the gramicidin dimer is incorporated in detergent micelles, the global correlation time is governed by the micelle tumbling rate. Molecular dynamics simulations reported an overall spheroidal shape of the gA-SDS complex, allowing the use of an isotropic diffusion model with a single τ_m value for all residues. The (T_1/T_2) ratio provides a way of estimating molecular correlation time because the short-range dynamics terms cancel out. We find similar T_1 and T_2 values, and consequently similar ratios for all backbone residues, which further indicates that a gramicidin dimer in a SDS micelle diffuses as a single unit³⁸.

The model-free analysis results are summarized in Table 2 and Figure 5. The different thermal energies at the different experimental temperatures account for the ~10 ns change in the molecular correlation times. While the τ_m value depends on numerous factors, including the SDS concentration, making comparisons with published data difficult, the values of 5–8 ns are typical of detergent-peptide complexes of similar size^{39,40}. We note that the correlation times, in principle, also can be complemented by using pulsed-field gradient diffusion experiments⁴¹.

To verify the extreme-narrowing limit condition, we performed the analysis using the additional correlation time as a free parameter. The τ_e values were consistent with fast bond reorientations, being <50 ps with few exceptions. When examined at different temperatures, the outliers were not identical; we therefore attribute them to experimental uncertainty. Furthermore, the extracted order parameters and associated errors were very similar in calculations that included the extra correlation time (Eq. 1). The high values of (T_1/T_2) ratios, being between ~4 (55 °C) and ~19 (30 °C), further attest to the τ_e values being on the order of several picoseconds¹³. The apparent T_2 values are primarily affected by low-frequency motion of the NH bond of interest and in addition to spin-spin relaxation mechanism, can have a contribution from chemical exchange¹³. The contribution from this extra term usually is detected by resonance broadening and/or anomalous low T_2 values; none of this was detected for gA in these experiments. Overall, the model-free analysis provides additional confirmation of a highly rigid gA molecule. The order parameter values typically vary between 0.84 and 0.92, indicating restricted motion of the peptide planes.

Discussion

Gramicidin channels^{42,43} present an appealing, small benchmark protein for evaluating theory and experiment within the context of lipid membranes and membrane-like

environments. We have examined the dynamics of the gA backbone and side chains in SDS micelles, using high-resolution solution NMR to determine the T_1 and T_2 relaxation times, the NOE enhancements and the deduced generalized order parameter S^2 as well as molecular tumbling rate τ_m . The preceding article¹ presents the results of MD simulations of the NMR observables and order parameter values and compares the predicted observables with the experimental results presented in this article.

The similar T_1 and T_2 times, and NOE enhancements for all of the backbone NH groups indicate that the gA channel structure is quite “stiff”, with only limited fluctuations about the mean atomic positions, even in the fluid micelle environment. The results for gA channels are similar to those for the core regions of several example small soluble proteins, such as lysozyme⁴⁴ and thioredoxin⁴⁵ or micelle-bound neuropeptide Y⁴⁶ and phospholamban⁴⁷, except that the gA channels lack the flexible loop regions that often are observed even for small soluble proteins. It is in this context notable that there seems to be nothing special about the NMR observables in the outer C-terminal half of each subunit. The relative stiffness extends even to the terminal Trp15 groups, indicating that Na^+ binding has little effect on the backbone structure and dynamics—in general agreement with previous solid-state NMR experiments in lipid bilayer environments^{11,48}.

Reflecting, perhaps, the stiff backbone structure with limited dynamics, the experimental NMR observables for the backbone are remarkably close to the predicted values from the MD simulations¹.

The NOEs for the Trp indoles vary more among the four side chains, indicating that the four side chains, though overall very similar, have different average environments and dynamics. While ^{15}N relaxation experiments are extensively employed for the investigation of backbone dynamics, fewer studies have focused on the behavior of tryptophan side chains. The measurements that exist have been performed primarily in water soluble proteins to analyze protein-protein interfaces⁴⁹ or investigate unfolded¹⁸ and partially folded⁵⁰ proteins.

A comparison between a tryptophan residue packed in a hydrophobic core and a water accessible one in thioredoxin revealed increased side chain mobility of the latter, manifested by a decrease of T_2 relaxation and NOE reduction⁴⁵. Similar trends can be observed for the Trp15 indole. While the reduction of the T_2 value is minor, there is a clear decrease in the heteronuclear NOE (Figure 4c) consistent with more extensive dynamics. Importantly, this enhanced motion is still largely restricted; in comparison, a tryptophan side chain in a flexible loop of lysozyme had NOE of 0.3 (at 600 MHz field)⁴⁴, while the one in an unfolded SH3 domain exhibited negative NOE¹⁸. Conversely, the T_2 relaxation appears to be more efficient for Trp9 and Trp11, and since the T_1 values are similar for all four indoles this may be an indication of slower internal motions of Trp9 and Trp11.

Comparing the predicted and observed NMR observables, the agreement for the indole side chains is somewhat worse than it was for the backbone, a result that presumably reflects the slow side chain dynamics that is not fully sampled in the MD simulations.

In conclusion, the experimental NMR observables serve as benchmarks for the MD simulations. The comparisons in the accompanying paper indicate that it is possible to use MD simulations to predict experimental NMR observables, at least in the case of the relatively stiff peptide backbone. Predicting side chain dynamics remains more challenging and will require long simulation times.

Acknowledgments

This work was supported in part by NIH grant GM070971 and by the Arkansas Biosciences Institute. The NMR facility was supported by NIH grants RR15569 and RR31154.

Abbreviations

DMPC	dimyristoylphosphatidylcholine
gA	gramicidin A
MD	molecular dynamics
SDS	sodium dodecylsulfate

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Figure 1. Physical characterization of the synthetic gA analogues. a: HPLC chromatogram; b,c: Mass spectra. Peak clusters correspond to $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions. Adjacent peaks within a cluster differ in mass by ± 1 due to the natural abundance of ^{13}C .

Figure 2. HSQC spectra of selectively labeled peptides. The panels correspond to different experiments using the peptides labeled in the rows in Table 1. The isotopically labeled residues are indicated.

Figure 3.

Spin-lattice (T_1) (a, b) and spin-spin (T_2) (c, d) relaxation decay curves for backbone (a, c) and side chain (b, d) of Trp9 (filled squares), Trp11 (open squares), Trp13 (filled circles) and Trp15 (open circles). Volume scale is identical between a, b and between c, d sets.

Figure 4. Spin-lattice (T_1) (a) and spin-spin (T_2) (b) relaxation times and steady-state NOE (c) at 30 °C (blue diamonds), 40 °C (red circles) and 55 °C (black squares).

Figure 5. Generalized order parameter S^2 at 30 °C (blue diamonds), 40 °C (red circles) and 55 °C (black squares).

Table 1Peptide sequences and ^{15}N label positions.

Data set ^a	Peptide	Sequence ^b
(a)	Gramicidin A	formyl- <u>VG</u> <u>A</u> <u>L</u> <u>A</u> <u>V</u> <u>V</u> <u>V</u> <u>L</u> <u>W</u> <u>L</u> <u>W</u> <u>L</u> <u>W</u> -ethanolamide
(b)	Gramicidin A (-) ^c	formyl- <u>VG</u> <u>A</u> <u>L</u> <u>A</u> <u>V</u> <u>V</u> <u>V</u> <u>L</u> <u>W</u> <u>L</u> <u>W</u> <u>L</u> <u>W</u> -ethanolamide
(c)	Gramicidin A	formyl- <u>VG</u> <u>A</u> <u>L</u> <u>A</u> <u>V</u> <u>V</u> <u>V</u> <u>L</u> <u>W</u> <u>L</u> <u>W</u> <u>L</u> <u>W</u> -ethanolamide
(d)	Gramicidin A	formyl- <u>VG</u> <u>A</u> <u>L</u> <u>A</u> <u>V</u> <u>V</u> <u>V</u> <u>L</u> <u>W</u> <u>L</u> <u>W</u> <u>L</u> <u>W</u> -ethanolamide

^aCorresponding panels in Figure 2.^bUnderlined residues represent D-amino acids. Residues in red were ^{15}N enriched.^cEnantiomer of gA, having opposite chirality.

Table 2

Molecular tumbling times, τ_m , of the gA dimer–SDS complex.

T/°C	τ_m/ns
30	17.0 ± 0.9
40	11.6 ± 1.0
55	6.6 ± 1.5