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A Proton T_1, T_2, and NOE Study of Relative Motion of the Indole Ring of Tryptophans in Gramicidin Analogs Incorporated into SDS Micelles

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The proton spin–lattice relaxation time, spin–spin relaxation time, and NOE of the indole ring NH proton of tryptophan residues have been determined for seven analogs of gramicidin A incorporated into SDS micelles. The data obtained indicate that the motion of the indole rings systematically decreases, proceeding from the aqueous interface to the interior of the micelle. © 1997 Academic Press

INTRODUCTION

The structure and function of peptides and proteins are often influenced by the dynamic characteristics of these biological systems. Furthermore, these dynamic characteristics can be determined by the environment into which the peptide or protein is placed. Within a peptide or protein, the position and dynamics of specific amino acid residues can also affect both structure and function. Membrane proteins have a significantly higher tryptophan (Trp) content than do soluble proteins (*1*). With membrane-bound peptides and proteins, tryptophan residues are frequently found at the interface between the aqueous and lipid environments (*1–5*). Through the ability to form hydrogen bonds at the aqueous–lipid interface (*1, 6*), tryptophan residues at this position provide a necessary structural anchor for that part of the peptide or protein that is near the aqueous environment. The polar nature of tryptophan can also contribute to the functional properties of the peptide or protein, as in the case of the ion-transport channel gramicidin.

The gramicidin family of linear polypeptides represents a biologically viable channel system of related peptides in which single amino acid residue replacement can cause changes in cation binding, cation selectivity, and transport (*7*). Gramicidin A, a naturally occurring analog that forms transmembrane channels, is a 15-residue hydrophobic peptide whose amino acid sequence is HCO-L ^{1}Val–^{2}Gly-L ^{3}Ala-D ^{4}Leu-L ^{5}Ala-D ^{6}Val-L ^{7}Val-D ^{8}Val-L ^{9}Trp-D ^{10}Leu-L ^{11}Trp-D ^{12}Leu-L ^{13}Trp-D ^{14}Leu-L ^{15}Trp-NHCH$_2$CH$_2$OH. When placed into lipid membranes or SDS micelles, gramicidin A forms right-handed, single-stranded $\beta^{6.3}$ helical dimer channels. The two gramicidin A monomers are joined at their NH_2 termini (*8–20*). Single-channel conductance studies have shown that there are differences in channel properties between gramicidin A analogs formed by single amino acid substitution (*21–24*). Amino acid substitution and side-chain orientation are thought to be important factors in determining the transport properties of the gramicidin channel (*7, 13, 24–33*).

The tryptophan residues of gramicidin appear to play an important role in modulating both structure and function of the peptide incorporated into model membranes. It has been suggested that the indole NH moieties may hydrogen bond to the aqueous interface or with the lipid molecules and this interaction may stabilize the gramicidin monomer in the lipid bilayer (*34–38*). The hydrogen bonding of the indole NH to the lipid bilayer surface has been suggested as playing a role in the transport of cations through the gramicidin channel (*28*). This is supported by the photodeactivation studies of the gramicidin channel (*39*). The importance of the number and position of the tryptophan residues in gramicidin on the incorporation into SDS micelles has also been demonstrated (*40*). However, the replacement of tryptophan by phenylalanine or tyrosine in gramicidin has been found to have no effect upon the backbone conformation of the peptide (*41*).

The dynamic nature of the gramicidin channel has been the subject of much interest. The libration of the carbonyl groups in the lumen of the channel and the relationship to ion transport have been investigated using a variety of theoretical and experimental techniques. Urry (*42*) suggested that the libration of the backbone carbonyl groups in the lumen is involved in the solvation of cations as they pass through the channel and plays an important role in the overall transport process. Theoretical calculations have also been used to study the dynamics of the backbone of the channel (*43–45*). The ^{15}N spin–lattice relaxation time (T_1) of the nitrogen atom at the ^{4}Leu position has been used to investigate the local dynamics about the ^{3}Ala–^{4}Leu linkage (*46*). Evidence was obtained that suggests a correlation between

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the local dynamics and ion transport through the channel. The backbone dynamics of gramicidin A in dimyristoylphosphatidylcholine (DMPC) bilayers have been studied using low-temperature solid-state ^{15}N NMR spectroscopy (*47*). Evidence for a difference in motion between ^{9}Trp and ^{11}Trp has been obtained with ^{2}H NMR spectroscopy of gramicidin A incorporated into DMPC bilayers (*48*). Motionally restricted tryptophan environments have been observed for gramicidin A incorporated into lipid bilayers composed of dioleoy-*sn*-glycero-3-phosphocholine (DOPC) using fluorescence spectroscopy (*49*).

We report the results of an investigation of the relative motion of the indole rings of the tryptophan residues in gramicidin A analogs incorporated into SDS micelles. Proton (^{1}H) spin–lattice relaxation time (T_1), spin–spin relaxation time (T_2), and NOE values were measured for *each* indole ring in seven analogs (gramicidin A, gramicidin B, gramicidin C, Phe-1 gramicidin A, Phe-1 gramicidin C, Gly-11 gramicidin A, and Gly-15 gramicidin A). The results of these experiments indicate that there is a systematic decrease in the motion of the indole ring of the tryptophan residues from ^{15}Trp (at the aqueous interface) to ^{9}Trp (at the interior of the micelles) for all of the analogs studied.

EXPERIMENTAL

The methods previously described in the literature (*50–52*) were used to obtain gramicidin A, B, and C from the commercially available gramicidin D (Sigma Chemical Co., St. Louis, Missouri), a mixture of the A, B, and C analogs. The Gly-11 and Gly-15 gramicidin A analogs were synthesized with an Applied Biosystems 431A peptide synthesizer (Foster City, California). The Phe-1 gramicidin A and Phe-1 gramicidin C were obtained using the semi-synthesis method (*32, 52*). Sodium dodecyl sulfate-d_{25} (SDS, 98% deuteration), trifluoroethanol-d_3 (TFE, 99% deuteration), and deuterium oxide (D_2O, 99.99%) were obtained from Cambridge Isotopes Laboratories (Cambridge, Massachusetts). The SDS was recrystalized from 95% ethanol. A 100 m*M* pH 6.5 phosphate solution was purchased from PGC Scientifics (Gaithersburg, Maryland). Approximately 25 to 50 m*M* solutions of the gramicidin analogs in TFE were added to 275 m*M* SDS (89% pH 6.5 buffer/11% D_2O v/v). The samples were sonicated in a Cole–Parmer 8851 sonicator for approximately 5 minutes, then 700 μl of the solution was transferred to a 4 mm NMR tube (Ultra High Precision, 535-PP, Wilmad, Buena, New Jersey). The final concentrations of the samples were approximately 3 m*M* in gramicidin analog, 250 m*M* SDS containing 80% pH 6.5 buffer/10% TFE v/v/v.

The ^{1}H NMR spectra were obtained with a Varian VXR-500S spectrometer. The T_1 values were determined using the inversion-recovery technique. Typical acquisition parameters used were 90° pulse time of 8.9 μs, 6000 Hz spectral window acquired in 16,384 data points and transformed in 32,768 using an equivalent of 0.1 Hz line broadening, 512 to 1024 FIDs for each spectrum. For each T_1 determination, 22 τ values (values ranged from 0.0001 to 12 s) were used with a total wait time (acquisition time and delay before the 180° pulse) of 15 s. The indole NH proton NMR signals of the tryptophan residues are particularly useful probes for studying the relative dynamic character of the indole rings since they are well resolved in the ^{1}H spectra (Fig. 1A). The indole NH peaks were assigned using the standard combination of the DQCOSY, TOCSY, and NOESY experiments. Figures 1B–1D clearly show the differences in relaxation time of the four indole NH protons of the tryptophan residues of Phe-1 gramicidin A. The T_1 values were calculated from the inversion-recovery data (peak intensity as a function of τ) using a nonlinear least-squares analysis. Each experiment was repeated a minimum of two times and the average of the T_1 values used for each indole hydrogen. The errors in the T_1 values range from 0.02 to 0.04 s.

Table 1 contains the spin–lattice relaxation times for the indole NH proton for each of the seven analogs as a function of position in gramicidin and temperature. Five of the seven analogs (i.e., gramicidin B with phenylalanine at position 11, gramicidin C with tyrosine at position 11, Phe-1 gramicidin C, Gly-11 gramicidin A, and Gly-15 gramicidin A) have only three tryptophan residues and, therefore, only three indole NH proton NMR signals. Consequently, there are only three entries for these residues (Table 1). The other blanks in Table 1 are the result of peak overlaps in the spectra which prevented the determination of individual T_1 values.

The T_2 experiments were performed with the Carr–Purcell (*53*), Meiboom–Gill (*54*) (CPMG) pulse sequence. Typical acquisition parameters used were 6000 Hz spectral window acquired in 16,384 data points and transformed in 32,768, using an equivalent of 0.2 Hz line broadening; 512 FIDs were accumulated for each spectrum; a spin-echo cycle time of 0.5 ms was used; and 17 T_2 relaxation time spectra were obtained for each T_2 measurement. The T_2 values obtained, although much shorter than the T_1 values as expected, are in agreement with the results of the T_1 measurements in that the T_2 relaxation time decreases from tryptophan 15 to tryptophan 9 for each analog. Typical values of T_2 for several analogs are gramicidin A (50°C) 15/0.024, 13/0.022, 11/0.021, and 9/0.018 s; gramicidin A (40°C) 15/0.017, 13/0.015, 11/0.015, and 9/0.013 s; Gly-11 gramicidin A (50°C) 15/0.028, 13/0.024, and 9/0.020 s; Gly-15 gramicidin A (50°C) 13/0.029, 11/0.028, and 9/0.025 s; and Phe-1 gramicidin A (50°C) 15/0.025, 13/0.020, 11/0.019, and 9/0.014 s. The differences in the linewidth of the indole NH peaks and, therefore, the intensity of these proton signals as

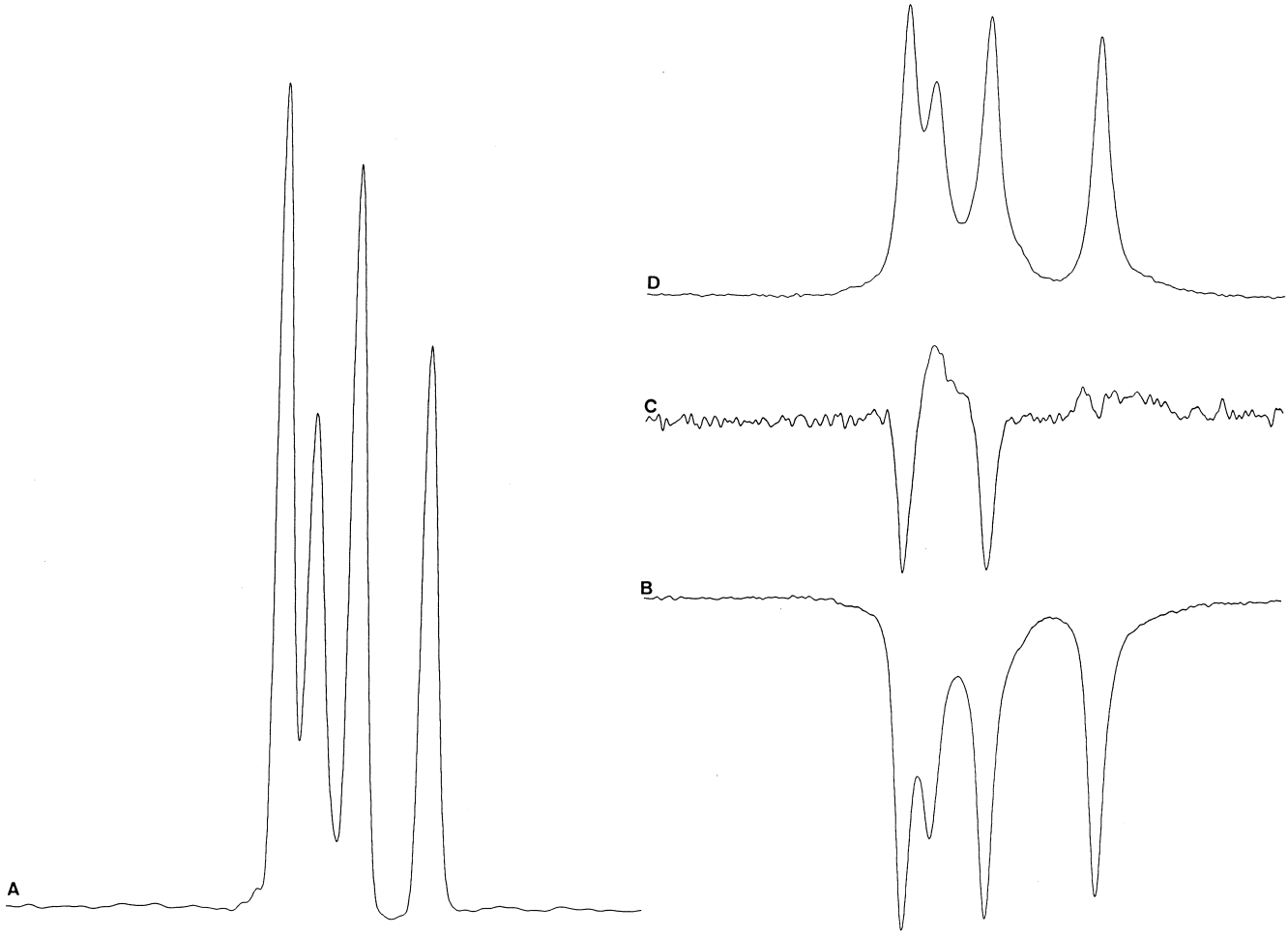

FIG. 1. (A) The proton spectrum of the Phe-1 gramicidin A analog at 311.38 K showing four well-resolved indole NH peaks for the four tryptophans in this analog. Spectra show the differences in the T_1 values of the four indole NH protons in this analog at the τ values used in the T_1 experiment of (B) 0.0001 s; (C) 0.8 s; and (D) 12 s. Spectrum (C) shows the obvious differences in the T_1 values.

observed in the spectra (Fig. 1A) are a reflection of the differences in the T_2 values.

Although very-low-power water presaturation was used in the T_1 and T_2 experiments, proton exchange is so slow (*55*) that this would not account for the difference in relaxation of the indole protons. Additional evidence for relating the difference in T_1 values of the indole NH protons to the motion of the indole rings instead of exchange was obtained by measuring the T_1 of one of the nonexchangable ring protons whose NMR signal was well resolved (i.e., not overlapped with another signal). With gramicidin A at 323.75 K, for example, the NH proton and the adjacent nonexchangable CH ring proton for residues 15 and 9 have T_1 values of 1.53 and 1.57 s, respectively, for 15 and 1.30 and 1.28 s, respectively, for 9.

The NOESY spectrum for gramicidin A in SDS micelles at 50°C is shown in Fig. 2. This spectrum was obtained with the following experimental parameters: 40 ms mixing time, spectral window of 6000 Hz acquired in a 4096 by 1024 matrix and transformed in 8192 by 2048 data points. A 40 ms mixing time was used to avoid spin-diffusion effects. The NOESY cross peaks are the same for each of the indole NH protons (see the ''circled'' region of the NOESY spectrum; the indole NH protons are in the region 9.6–9.9 ppm and the indole ring protons are in the range 7.0–7.5 ppm). These cross peaks arise from the interaction with the protons on tryptophan carbon atoms adjacent to the indole NH proton. These protons have the same fixed distance on each tryptophan ring. No other cross peaks involving the indole NH protons are observed. The absence of other cross peaks is consistent with the three-dimensional structure of gramicidin determined by the combination of NOESY distance restraints, relaxation-matrix calculations, and molecular modeling. Consequently, differential relaxation caused by other

TABLE 1
T_1 Values of the Indole Protons of Gramicidin Analogs

Residue	Temp. (K)	GramA T_1 (s)	GramB T_1 (s)	GramC T_1 (s)	Phe1A T_1 (s)	Phe1C T_1 (s)	Gly15A T_1 (s)	Gly11A T_1 (s)
15	323.75	1.53	1.61		1.47	1.43		1.46
	319.38	1.42	1.54		1.36	1.28		1.45
	316.09	1.27	1.45	1.23		1.15		1.37
	311.38	1.12	1.33	1.07		0.95		1.25
	307.20	0.96	1.17	0.93		0.81		1.12
	302.58							0.97
13	323.75	1.58	1.64		1.46	1.44	1.65	1.49
	319.38	1.34	1.55		1.35	1.27	1.49	1.42
	316.09	1.24	1.47	1.19	1.28	1.09	1.34	1.31
	311.38	1.09	1.30	1.04		0.90	1.06	1.19
	307.20	0.94	1.15	0.90		0.77		1.06
	302.58							0.92
11	323.75	1.42			1.29		1.34	
	319.38	1.24			1.20		1.26	
	316.09	1.11			1.15		1.16	
	311.38	0.99					0.94	
	307.20	0.89						
	302.58							
9	323.75	1.30	1.36		1.22	1.14	1.24	1.22
	319.38	1.17	1.26		1.17	1.02	1.16	1.17
	316.09	1.04	1.17	0.97		0.9	1.08	1.06
	311.38	0.90	1.06	0.89		0.75	0.88	0.97
	307.20	0.80	0.95	0.77		0.64		0.88
	302.58							0.77

protons in the molecule that might not be at the same distances from the indole NH proton on the four tryptophan rings does not appear to be present. Since the SDS used to form the micelles is deuterated, intermolecular interactions play only a minor role in the relaxation of the indole NH protons.

DISCUSSION

It is important to have an understanding of the internal motions within proteins and peptides since they have an effect upon the structure–function relationship (*49, 56–61*). The data in Table 1 reveal several general trends concerning the dynamic behavior of the indole ring of each of the tryptophan residues in the gramicidin analogs incorporated into SDS micelles. From the temperature dependence of the T_1 values (*62*), ΔG^* values of activation for the tryptophan motion of about 18.5 kcal/mol were calculated. This relatively large free energy of activation for tryptophan motion is in agreement with the results of fluorescence spectroscopy which indicated restricted motion of the tryptophan residues in the gramicidin channel (*49*).

Two other trends are revealed through a study of the effects of temperature and position in the channel on the motion of the Trp side chains. A decrease in the value of T_1 is observed with a decrease in temperature. This relationship is indicative of a decrease in motion of the indole ring as the temperature decreases according to the theory of spin–lattice relaxation (T_1) for relatively short correlation times (*63*). Within each analog, there appears to be a systematic decrease in the T_1 value of the indole rings from position 15 (longest T_1) at the aqueous interface to position 9 (shortest T_1) in the interior of the micelle. This decrease in T_1 indicates a slowing of the motion of the indole rings as they are buried further and further into the interior of the micelle. Greater motion at the aqueous interface would seem to be in agreement with the ^{15}N NMR characterization of the backbone librations of gramicidin A (*47*). However, Koeppe (*48*) found in a ^{2}H NMR study of the orientation of ^{9}Trp and ^{11}Trp of gramicidin A in oriented model membranes that ^{9}Trp appeared to have greater motion than ^{11}Trp. It is not obvious as to why the two experiments appear to contradict one another. The results of the two experiments may well represent differences between the SDS and lipid environments as well as differences in interaction between the tryptophan side chains and the SDS and lipid environments.

While relaxation in biological systems is a complex phenomenon, the data presented here for the indole NH proton on tryptophan residues separated by only about 10 Å and in

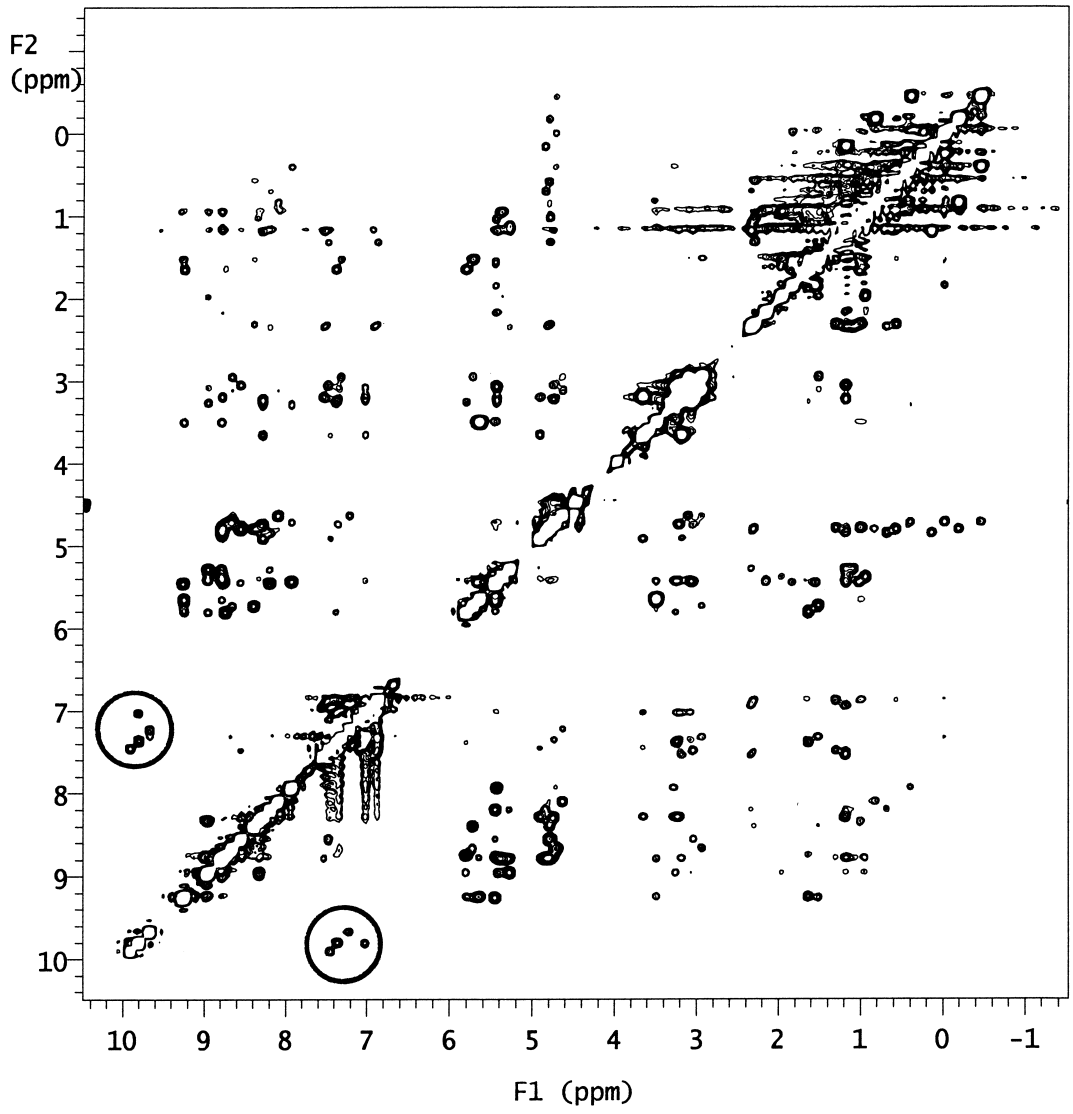

FIG. 2. The NOESY spectrum of gramicidin A. The peaks within the circles represent the indole NH protons (region, 9.6–9.9 ppm) with indole ring protons (region, 7.0–7.5 ppm).

the same environment indicate that the difference in T_1 and T_2 values for these protons is the result of a decrease in the overall motion of the indole ring proceeding from the aqueous interface to the interior of the micelle.

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