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Direct observation of differential UV photolytic degradation among the tryptophan residues of gramicidin A in sodium dodecyl sulfate micelles

Steve McKim and James F. Hinton *

Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701 (USA)

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Gramicidin A, incorporated into sodium dodecyl sulfate micelles, was exposed to ultraviolet light and discovered by two-dimensional (TOCSY) NMR spectroscopy to undergo differential photolytic degradation. The four tryptophan residues of gramicidin A were found to be unequally sensitive to ultraviolet radiation. Tryptophan 9 was the most sensitive to ultraviolet photolysis, while tryptophan 11 was the least sensitive. Tryptophans 13 and 15 have approximately the same susceptibility to photolytic degradation by the ultraviolet light. Rate constants for the photolytic degradation of the four tryptophan residues were obtained from the dependence of the TOCSY spectrum upon the time of photolysis.

Introduction

Gramicidin, an antibiotic produced by the bacterium *Bacillus brevis* during sporulation [1], is a small peptide composed of 15 alternating D-L amino acid residues. Gramicidin can function as a monovalent cation conducting channel when incorporated into lipid bilayers or membranes [2–5]. A mixture of gramicidins is produced by *Bacillus brevis*, with the three major products being gramicidins A, B, and C. Gramicidin A is composed of the following sequence of amino acids: formyl-L-Val^1-Gly^2-L-Ala^3-D-Leu^4-L-Ala^5-D-Val^6-L-Val^7-D-Val^8-L-Trp^9-D-Leu^{10}-L-Trp^{11}-D-Leu^{12}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15}-ethanolamine. Gramicidins B and C differ from gramicidin A only at position 11, with gramicidins B and C having phenylalanine and tyrosine residues at this position, respectively [6–9]. The functional form of the channel traversing the bilayer is a 26 Å long, 4 Å diameter structure resulting from the hydrogen bond linkage of two gramicidin monomers at their NH_2 terminal ends [10–16].

When the gramicidin A channel is exposed to ultraviolet (UV) light, the tryptophan residues are photochemically altered in such a way as to make the channel essentially impermeable to monovalent cation transport [17–19]. Differential UV photolysis has been reported for the four tryptophan residues of calf γ-II crystallin [20] and for several di- and tripeptides containing one tryptophan residue [21,22]. It has also been suggested that differential photolysis occurs among the four tryptophan residues of gramicidin A [18,23]. We wish to report the results of a study which provides direct evidence for differential photolytic degradation of the four tryptophan residues of gramicidin A.

Materials and Methods

Gramicidin A, isolated from gramicidin D (80% gramicidin A, 5% gramicidin B, and 15% gramicidin C, Sigma, St. Louis, MO) by HPLC, sodium dodecyl sulfate (SDS) (Bio-Rad, Richmond, CA) and D_2O (99.96% D, 10 × 0.7 ml vials, Cambridge Isotope Laboratories, Woburn, MA) were used without further purification. Fully deuterated SDS (SDS-d_{25}) (Cambridge Isotope Laboratories) and L-kynurenine (United States Biochemical, Cleveland, OH) were recrystallized from 95% ethanol.

The 2D (TOCSY) NMR spectra [24] were acquired at 55°C for a 6 mM gramicidin A/0.25 M SDS sample, prepared with an aqueous pH 6.50 buffer (made from a pH 6.50 pHydrion Buffer Capsule, Micro Essential Laboratory, Brooklyn, NY) using a modification of the method described by Arseniev et al. [25]. The gramicidin A/SDS solution was 90% H_2O/10% D_2O, the standard solution for 2D NMR spectra. After the initial TOCSY spectrum of this sample was recorded, it was removed from the NMR tube, placed in a quartz

* Corresponding author. Fax: +1 (501) 5754049.

cuvette and irradiated at 25°C with a 75 Watt UV lamp (Photon Technology International Model LPS-220 Xe Arc Lamp, Princeton, NJ) which typically produces a continuous spectrum over a range of 250–600 nm [26]. The lamp was equipped with a water filter. After photolysis, the sample was placed back into the NMR tube. Additional TOCSY spectra were acquired after the sample had been photolyzed for 8, 16 and 24 total hours. A dark control experiment was done on an unirradiated gramicidin A/SDS samples using 2D NMR which showed the sample to be stable for a period of up to six months. The volume of each of the cross-peaks representing the four indole N-H protons of the tryptophan residues of gramicidin A was obtained using Varian VNMR software (Varian, Palo Alto, CA). These cross-peaks are related to the gramicidin concentration. Two independent NMR and irradiation experiments were performed on two separate 6 mM gramicidin A/0.25 M SDS samples. All NMR spectra were obtained with a Varian VXR-500S NMR Spectrometer (Varian, Palo Alto, CA).

UV spectroscopy was used to study the absorption profile of the tryptophan residues of gramicidin A after extended durations of UV photolysis. A 6 mM gramicidin A sample was prepared again for these studies [25]. The UV absorption spectra of this gramicidin containing sample were obtained at 25°C, over the range of 200–450 nm, using a Hewlett Packard 8452A Diode Array Spectrophotometer (Hewlett Packard, Palo Alto, CA). For a comparison with the photodegradated gramicidin A sample, a UV spectrum of 24 mM L-kynurenine in 0.25 M SDS was obtained. This sample was prepared by dissolving the appropriate amount of the amino acid into an SDS/pH 6.5 buffer solution and vortexing until clear.

Circular dichroism spectroscopy was used to determine if gramicidin A retained its original conformation in SDS micelles after prolonged exposure to UV radiation. Circular dichroism spectra of a 6 mM gramicidin A/SDS sample before and after 32 h of UV photolysis were recorded (Jasco J-710 Spectropolarimeter, Japan Spectroscopic, MD), at 25°C, over a range of 200 to 300 nm with a 1 s time constant, spectral steps of 0.2 nm, and a scan rate of 50 nm/min. Six scans were acquired and averaged.

Results and Discussion

In order to determine if the tryptophan residues of gramicidin A were affected equally by UV irradiation or whether differential degradation occurred, rate constants were determined for the photolytic degradation of gramicidin A in SDS micelles from the cross-peaks representing the indole protons of the four tryptophan residues in the 2D (TOCSY) NMR spectra. The signals (cross-peaks) for the four tryptophan indole protons are very well resolved in the TOCSY spectrum, as shown in Fig. 1. The assignment of the cross-peaks of Arseniev et al. [25] has been confirmed in this laboratory by complete analysis of the DQCOSY, TOCSY and NOESY NMR spectra of gramicidin A in SDS micelles. TOCSY spectra of the indole N-H region are shown in Fig. 1 after: (A) no photolysis, (B) 8 h, (C) 16 h, and (D) 24 h of UV photolysis. The volume of the indole N-H peak of each tryptophan residue can be seen to diminish with increasing exposure to UV irradiation. This is consistent with photochemical degradation of the tryptophans. However, it is apparent that the tryptophans are not equally affected by UV irradiation. The cross-peak in the TOCSY spectra for Trp 9 appears to diminish at a rate greater than that of the other Trp residues. Although Trp 9 appears to be absent in spectrum D, after 24 h of UV photolysis, it can be observed using a lower spectral threshold.

Since the volume of a cross-peak is proportional to the concentration of the species producing it, a first-order rate constant was determined for the degradation of the indole N-H peak of each Trp residue from the relationship between the logarithm of the volumes as a function of UV photolysis time. These plots can be seen in Fig. 2. The first-order degradation rate constant (slope) and half-life of the indole N-H proton of each Trp is contained in Table I. These data show differential UV photolytic degradation of the four tryptophan residues. Trp 9 is the most susceptible to UV photolysis and Trp 11 is the most resistant. The effect of UV radiation on Trps 13 and 15 appears to be approximately the same.

It has been reported that the photooxidation of tryptophan can occur through the following mechanism [17,27–29]: (1) absorption of a photon by the indole ring, causing an indole electron to dissociate from the tryptophan with subsequent electron solvation by the aqueous phase, (2) the resulting Trp^+ radical, being highly acidic, loses H^+ (microsecond time scale) from the indole nitrogen, and (3) the neutral Trp radical reacts with O_2 (sub-millisecond time scale) to produce various stable photochemical products. Thus, the accessibility of the neutral Trp radical to O_2 dissolved in the aqueous phase should be an important factor in determining the rate of tryptophan photolysis. Therefore, Trp residues closer to the aqueous-membrane interface might be more vulnerable to photolysis than those Trp residues buried in the micelle [20].

Additional factors, determined from the photolytic studies of di-and tripeptides, containing only one Trp residue, include the nature of the amino acid adjacent to Trp and whether the carboxyl or amino group of Trp participates in the peptide bond [21,22]. Peptides in which the amino group of Trp forms the peptide linkage photolyzed at a much faster rate than peptides where the carboxyl group of Trp was part of the

peptide bond. It was also observed that the smaller the amino acid at the amino terminus of the dipeptide, the faster the photolysis rate [22]. The mechanism thought to be responsible for this increased photolysis rate involves the production of a lactam from the amino acid on the amino terminal end and tryptophan. The smaller the amino acid linked to Trp, the faster the production of the lactam due to less steric hindrance [22]. The lactam still retains the indole ring from the tryptophan residue [22,30]. This lactam formation mechanism does not require the presence of oxygen [22,30]. Although these studies centered on di- and

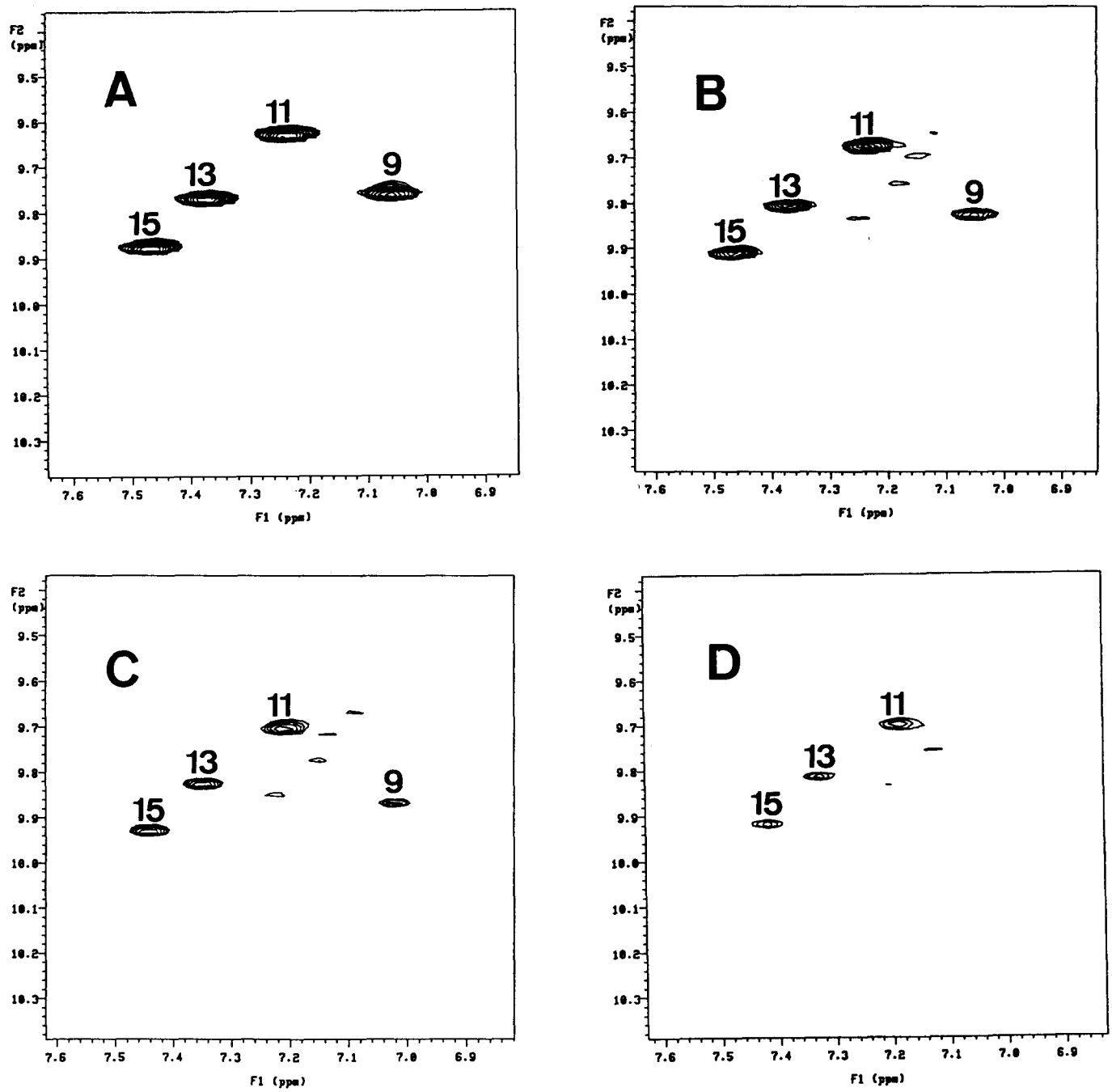

Fig. 1. TOCSY NMR spectra, at 55°C, of the indole N-H region of a 6 mM gramicidin A/0.25 M SDS-d_{25} sample after: (A) no photolysis, (B) 8 h, (C) 16 h, and (D) 24 h of UV photolysis. 4K×256 data points, a mixing time of 70 ms and water suppression were used to acquire the spectra. The same vertical scale and threshold were used in each spectrum. In order to photolyze the sample, it was removed from the NMR tube, placed in a quartz cuvette and photolyzed with a 75 Watt UV lamp equipped with a water filter for the desired duration. After photolysis, the sample was placed back into the NMR tube and the TOCSY spectrum of the sample was acquired. The separate resonances of Trps 9, 11, 13, and 15 in each spectrum are indicated by the appropriate number above the peak. Trp 9 appears to be absent in spectrum D, but can be found using a lower spectral threshold value.

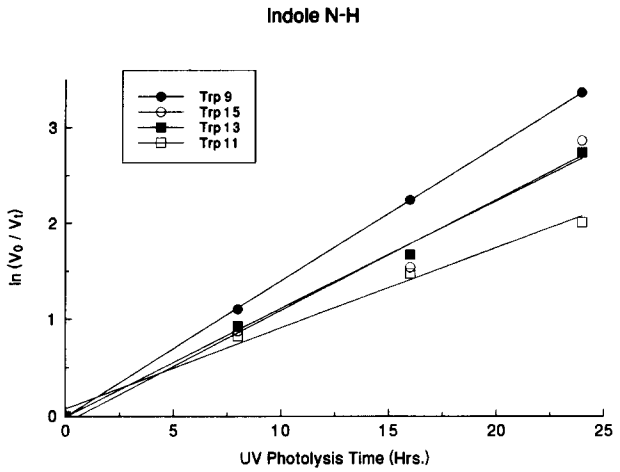

Fig. 2. Plots of the logarithm of the indole N-H proton peak volumes of each tryptophan residue (see Fig. 1) with respect to UV photolysis time. The slopes of the lines are the first-order degradation rate constants of the indole N-H proton of each respective tryptophan residue (see Table I). V_0 is the initial volume of the peak before photolysis and V_t is the volume of the peak at time t. Peak volumes were obtained using Varian VNMR software.

tripeptides, it has been speculated that the photolysis rate of tryptophan in proteins may also be dependent on the nature of the amino acid linked to the amino terminal end of Trp [31]. The conditions employed in the photolysis experiments with gramicidin, although differing from those used by Tallmadge and Borkman [20] and Dillon [30] in terms of pH, lamp intensity (the irradiation temperature in [20] was the same, 25°C, as that used in this study), protein concentration and solution composition (their proteins and/or peptides were not incorporated into micelles) yielded results which can be explained in terms of the mechanisms proposed in these two studies [20,30].

The structure of gramicidin A in SDS micelles, as deduced from two-dimensional ^{1}H NMR, shows Trps 13 and 15 to be close to the aqueous membrane interface, with Trps 9 and 11 being found deeper within the micelle (Ref. 25, and confirmed in this laboratory). This could explain why Trps 13 and 15 are photolyzed at approximately the same rate, their respective radicals having roughly equal access to aqueous O_2. Trp 11 has the slowest photolysis rate of the four tryptophan residues. Because Trp 11 is located deeper within the interior of the micelle than Trps 13 and 15, its radical may not be as accessible to aqueous O_2. Although Trp 9 is found at about the same depth within the micelle as Trp 11, it was observed to be the most susceptible to UV irradiation. This apparent anomaly may be related to a mechanism involving the lactam formation reaction.

TABLE I

The UV photolytic degradation rate constant and half-life of each indole N-H proton of the four tryptophan residues of gramicidin A

Residue	Rate constant k (h^{-1})	Half-life $t_{\frac{1}{2}}$ (h)
Trp 9	0.14±0.004	5.0
Trp 11	0.08±0.002	8.7
Trp 13	0.11±0.003	6.3
Trp 15	0.12±0.003	5.8

The amino acids with which the amino group of the four Trps of gramicidin A participate in a peptide bond are: Val 8-Trp 9, Leu 10-Trp 11, Leu 12-Trp 13, and Leu 14-Trp 15. All the Trp residues of gramicidin A are linked via their amino group to Leu residues except Trp 9, which is linked to a smaller Val residue. Although the photolysis rate of the photooxidation reaction involving Trp 9 should be roughly equal to that for Trp 11, the formation of the lactam involving Trp 9 would be formed faster than that of the other Trp residues. This faster lactam formation may compensate for the relative solvent inaccessibility of Trp 9 to O_2 within the micelle.

Fig. 1 appears to contain evidence which is consistent with lactam formation. After the 6 mM gramicidin A/SDS sample was subjected to 8 h of UV photolysis (spectrum B), additional cross-peaks appear around the diminished indole N-H signals of tryptophan. These peaks may arise from the lactams formed upon photolysis of the Trp residues. As expected, their chemical shifts are only slightly different from those of the tryptophans, since these lactams contain the intact indole ring and the indole N-H protons. These peaks can be seen after 16 h of photolysis (spectrum C), but appear to disappear after 24 h of UV exposure (spectrum D), indicating that the lactams themselves are photolabile.

One of the products, or family of products, known to be produced by the photolysis of tryptophan is kynurenine and its derivatives [17,19,32–36]. To determine whether kynurenine was present as a photoproduct of the tryptophan residues of gramicidin A, a 6 mM gramicidin A/SDS solution was subjected to varying periods of UV irradiation. During the photolysis, aliquots of this solution were withdrawn and diluted, and the UV spectra obtained. Additionally, the UV spectrum of a 24 mM kynurenine/SDS solution was also obtained. Displayed in Fig. 3 are the UV spectra of: (A) gramicidin A, before photolysis, and (B) kynurenine. The remaining UV spectra are those of the gramicidin A sample after UV irradiation. After 12 h of UV irradiation, the UV spectrum of the gramicidin A/SDS sample appears to indicate the presence of tryptophan ($\lambda_{max} = 280$ nm). This tryptophan absorbance at 280 nm of this gramicidin A/SDS solution was still significant even after the sample had been photolyzed for 32 total hours. However, the absorption profile of the lactam should be very similar to that of

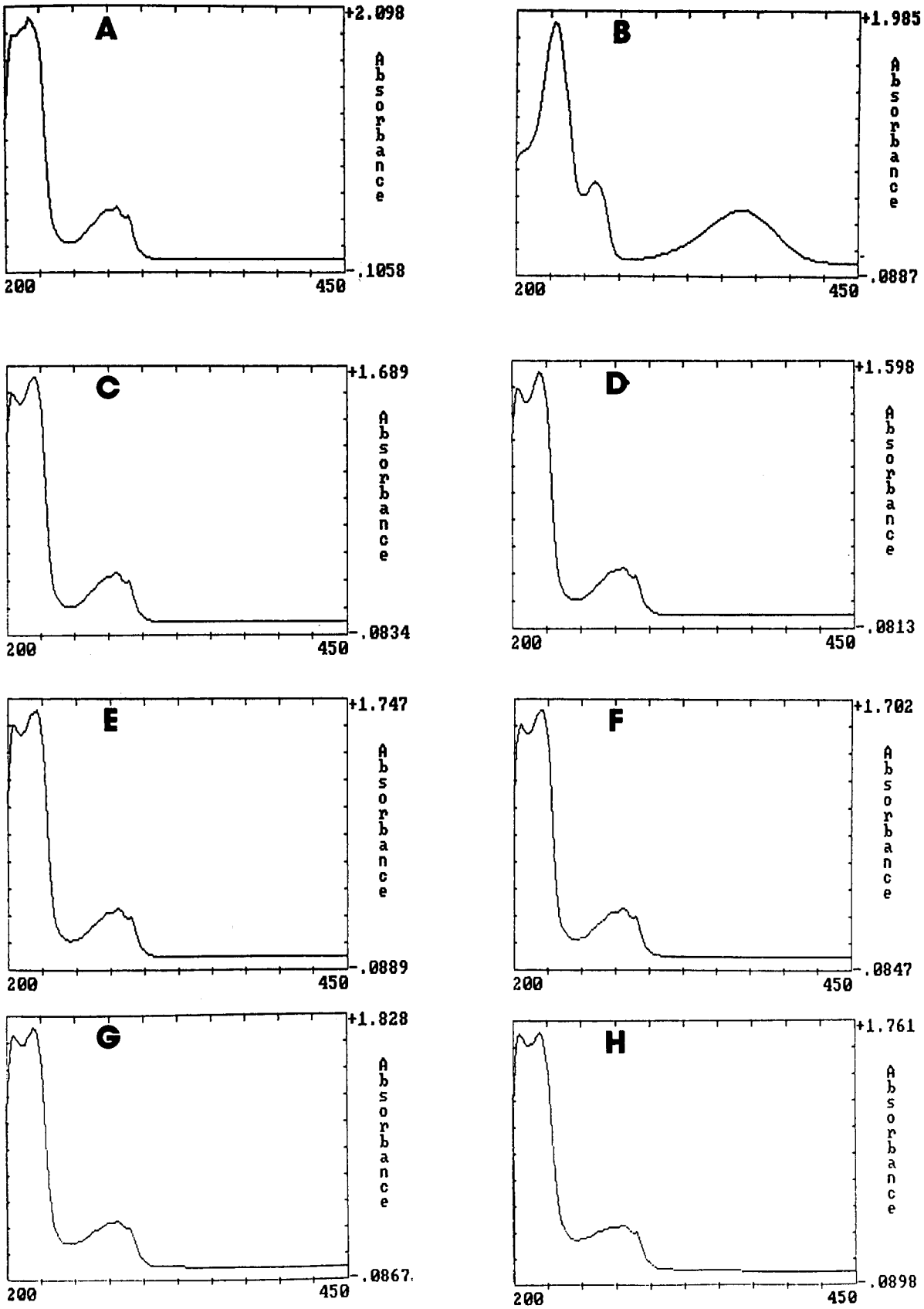

Fig. 3. UV spectra, at 25°C, of diluted aliquots of a: (A) 6 mM gramicidin A/0.25 M SDS, (B) 24 mM kynurenine/0.25 M SDS. The remaining spectra are of the 6 mM gramicidin A sample after exposure to: (C) 5 min, (D) 10 min, (E) 30 min, (F) 1 h, (G) 6 h, and (H) 12 h of UV photolysis. The 6 mM gramicidin A sample was subjected to varying durations of UV radiation. During photolysis, aliquots were withdrawn, diluted, and UV spectra obtained on a diode-array UV spectrometer over the range of 200–450 nm.

tryptophan, since it contains the Trp indole ring [22]. This apparent absence of significant change in the UV spectra of gramicidin A after exposure to long durations of UV photolysis lends additional evidence to lactam formation. Kynurenine was not detected as a byproduct of the UV photolysis of tryptophan in any of the gramicidin spectra in Fig. 3. However, kynurenine, like tryptophan, is known to be sensitive to UV photolysis, perhaps more so [32]. Thus, its absence from the UV spectra of Fig. 3 could be due to its photolytic susceptibility and/or its spectrum being hidden by other photooxidative products of tryptophan. Some of these photooxidative products absorb in the same wavelength region as tryptophan [18].

It has been speculated that differential UV photolysis of the four tryptophan residues of gramicidin A could occur through a quenching process [18,19]. It has been suggested that quenching may occur between Trps 9 and 15, which would tend to hinder the photolytic activity of these particular residues [18,19]. Presumably, this quenching would be due to the stacking of the indole moieties of Trps 9 and 15 of gramicidin A [23]. However, quenching can be ruled out as a major factor in the differential photolysis of the four tryptophan residues of gramicidin A. If quenching were the predominant mechanism, the rates of photolysis of Trps 9 and 15 would be slower than the rates of Trps 11 and 13. However, this was not observed. The orientation of Trps 9 and 15 with respect to each other can

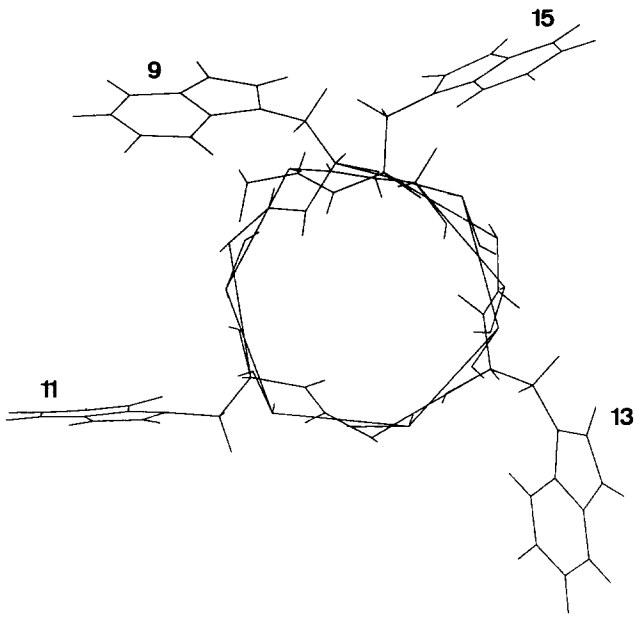

Fig. 4. Molecular modeling representation of a gramicidin A monomer in SDS micelles based upon the structure, obtained in this laboratory, from NOESY determined distance restraints and molecular modeling. The view is through the channel lumen. Only the peptide backbone is shown with all the side chains removed except for the four tryptophan residues. Trps 9, 11, 13 and 15 are indicated with the appropriate numbers by the residues.

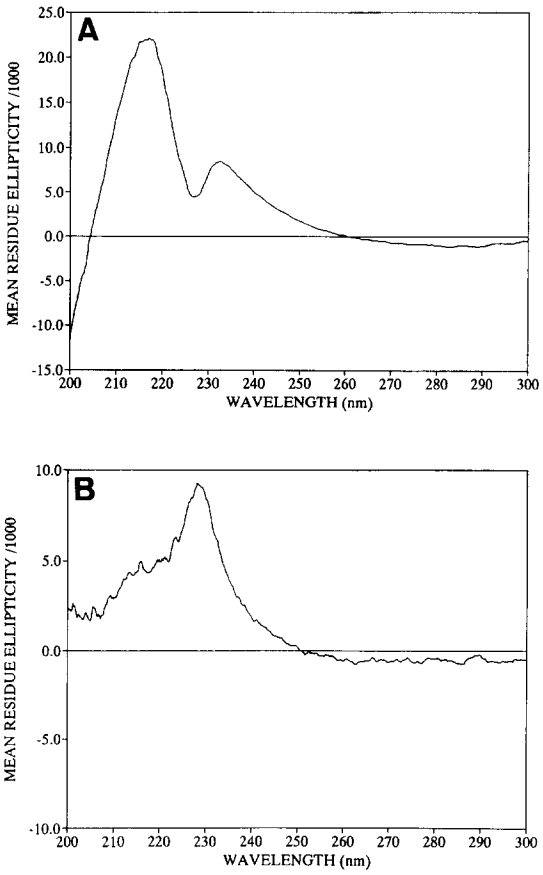

Fig. 5. Circular dichroism spectra at 25°C of a 6 mM gramicidin A/0.25 M SDS-d_{25} sample after: (A) no photolysis and (B) 32 total hours of UV photolysis. The CD spectra were obtained on a spectropolarimeter over the wavelength range 200–300 nm with a 1 s time constant, spectral steps of 0.2 nm and a scan rate of 50 nm/min. 6 scans were averaged.

be seen in Fig. 4, which is a molecular modeling representation of a gramicidin A monomer in SDS micelles, based upon the structure obtained from NOESY determined distance restraints and molecular modeling (Ref. 25 and confirmed in this laboratory). Only minor stacking could be said to occur between Trps 9 and 15, thereby producing a minimal quenching effect between these two residues.

The conformation of the gramicidin A channel in SDS micelles is known to consist of two right-handed, single-stranded $^{6.3}\beta$ helices (6.3 residues per turn), linked by hydrogen bonds at their NH_2 terminal ends. This conformation yields a characteristic circular dichroism (CD) spectrum in SDS micelles and lipids [25,37]. To determine if this helical structure was still retained after a long exposure to UV light, the CD spectrum was obtained for a 6 mM gramicidin A/SDS sample subjected to 32 h of UV irradiation. The result is displayed in Fig. 5, with the CD spectrum (A) being that of an unphotolyzed 6 mM gramicidin A/SDS sample and CD spectrum (B) that of the photolyzed 6 mM gramicidin A/SDS sample. From a comparison of

the two CD spectra, the gramicidin A channel in SDS micelles no longer appears to be in its $^{6.3}\beta$ helical (unphotolyzed) conformation after long exposures to UV light. This result is due either to a conformational change or to the photochemical destruction of the channel.

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