CHEMICAL AND BIOLOGICAL PROPERTIES OF FORMYL GRAMICIDINS S

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The mono- and diformyl gramicidins S have been prepared. Monoformyl gramicidin S retains about 50% of the expected biological activity and the diformyl derivative is inactive. It is therefore, conceivable that both the amino groups equally contribute to the biological activity of the antibiotic. However, monoformyl gramicidin S has been found to form aggregate and this aggregate is more stable under acidic conditions rather than in neutral or alkaline solutions. Denaturing agent urea has been found useful in dissociating the aggregate. The aggregating ability of formyl peptides is at least be due to their formyl groups.

The chemistry and biology of the cyclic peptide antibiotic gramicidin S has been reviewed[1]. While working on the structural aspects required for the biological activity of polymyxins[2] formyl polymyxins have been found[3] to exhibit selective aggregation phenomenon. In our studies on monoformyl gramicidin S it has been realized that this derivative has aggregating properties similar to that of formyl polymyxins. The results of such investigation on gramicidin S together with the biological activity of formyl derivatives are communicated in this paper. As found in formyl polymyxins and gramicidin DUBOS[4], a naturally occurring peptide antibiotic, monoformyl gramicidin S shows aggregation which seems to be unique among formyl peptides.

Materials and Methods

Gramicidin S sulfate was a gift from Prof. L. K. RAMACHANDRAN, Department of Biochemistry, Osmania University, Hyderabad, India. Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Sweden.

Amino groups were determined by a literature method[5] and the formyl groups were determined as formyldinitrophenylhydrazine[6]. The ninhydrin negative compounds on electrophoresis strips were detected by RYDON's method[7]. Colorimetric measurements were carried out on a Coleman Junior Spectrophotometer.

The antibacterial activity of gramicidin S and its derivatives was assayed by the tube dilution technique using *Streptococcus faecalis, Bacillus subtilis, Escherichia coli, Micrococcus pyogenes* and *Staphylococcus aureus*. The activity is expressed as the concentration required for minimal growth inhibition of bacteria.

Preparation of Monoformyl Gramicidin S (F_1GS)

Gramicidin S sulfate (100 mg) was formylated[8] with formic acid - acetic anhydride, 3: 1 (1 ml) for 4 hours at room temperature. Cold water (0.5 ml) was added to the reaction mixture and it was evaporated to dryness. The residue was suspended in 0.1 N $NaHCO_3$ (0.5 ml), centrifuged and washed twice with water (2 ml). Yield 90 mg, mp. 273~274°C.

Purification of Formylated Gramicidin S

The sample was purified on a Sephadex G-25 column (40×1.1 cm) with aqueous alcohol (50%) as eluent to yield two peak materials. The two components were pooled and lyophilized. On a dry weight basis the peak materials A and B were found to be in the ratio of 40% and 60%, respectively. Analy-

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sis for amino groups showed the presence of 1.02 and 1.05 amino groups/mole, respectively, in compounds A and B. Elemental analysis of these fractions gave the following data. Component A: *Anal.* Calcd. for $C_{61}H_{92}N_{12}O_{11}$: C 62.65, H 7.93, N 14.37. Found: C 62.98, H 7.54, N 14.32. Component B: *Anal.* Calcd. for $C_{61}H_{92}N_{12}O_{11}$: C 62.65, H 7.93, N 14.37. Found: C 62.82, H 7.62, N 14.38. Rechromatography of fraction A on Sephadex G-25 yielded fractions corresponding to A and B but with differential proportions.

F_1GS has been also chromatographed in cold and at room temperatures using solvent systems 0.01 N NaOH in 50% alcohol and formic acid - acetic acid - water (4: 15: 180) to study the effect of temperature and pH on the formation of the two peaks.

Paper electrophoresis on Whatman 3MM paper strips (4×46 cm) in formic acid - acetic acid - water (4: 15: 180) with 400 V for 4 hours of monoformyl gramicidin S before purification on Sephadex G-25 showed the presence of two ninhydrin positive bands. Electrophoresis of F_1GS under the above conditions but on paper strips saturated with 6 M urea gave a single band.

Preparation of Diformyl Gramicidin S (F_2GS)

Gramicidin S sulfate (120 mg, 100 μmole) dissolved in 40% alcohol was treated with sodium bicarbonate (16 mg, 200 μmole) for 10 minutes at room temperature and then the contents were evaporated to dryness under vacuum. Formylation of this residue was achieved under the conditions used for the preparation of F_1GS. Yield 95 mg, mp 270~272°C. This sample contained 0.03 amino groups/mole. *Anal.* Calcd. for $C_{62}H_{92}N_{12}O_{12}$: C 62.19, H 7.74, N 14.04. Found: C 62.05, H 7.62, N 13.98. Chromatography on paper and paper electrophoresis showed the absence of ninhydrin positive component.

Results and Discussion

At least one of the two amino groups is essential for the biological activity of gramicidin S (Fig. 1). This is evident from the data shown in Table 1. Diformyl gramicidin S having no free amino group has about 5% of the activity of the original antibiotic. On the other hand monoformyl gramicidin S with a free amino group showed nearly 50% of the expected activity (Table 1). It seems therefore, that the two cations equally contribute to the activity of gramicidin S. Earlier investigations have shown that acylation[9] of both the amino groups that neutralizes the cationic charge on the molecule would inactivate the antibiotic and monosubstitution results in partial loss of activity. On the contrary alkylation or guanidation[9] of the amino groups of ornithine residue that retain the positive charge on the molecule had no considerable effect on the activity.

Fig. 1. Structure of gramicidin S.

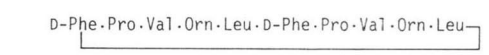

Table 1. Antibacterial activity of gramicidin S (GS) and its derivatives.

Organism	Minimal inhibitory concentration (μg/ml)*			
	GS	F_1GS	Peak A	F_2GS
Bacillus subtilis	5.2	11.2 (46.5)	11.3	95 (5.4)
Streptococcus faecalis	0.5	0.96 (52.0)	0.95	75 (0.7)
Escherichia coli	3.0	7.2 (41.0)	7.20	91 (3.0)
Micrococcus pyogenes	1.0	1.80 (55.0)	1.70	72 (1.4)
Staph. aureus	5.6	12.1 (46.0)	12.22	108 (5.4)

* Values in parentheses are percentages.

The aggregation of monoformyl gramicidin S was noticed during purification and characterization steps. Peaks A and B obtained on Sephadex G-25 chromatography of F_1GS are chemically indistinguishable and since peak A runs ahead of gramicidin S as a high molecular weight material it has been designated as aggregate of F_1GS. Further evidence is obtained by electrophoretic analysis of F_1GS. Electrophoresis of F_1GS on paper strips containing no urea gives two distinct ninhydrin positive bands.

Whereas electrophoresis of F_1GS on paper strips containing 6 M urea results in the disappearance of a band close to the origin. The slower moving band close to the origin found on paper strips containing no urea could thus be an aggregate of F_1GS and possibly this runs as peak A material on Sephadex G-25 column. The peak A material is quite unstable since rechromatography of it yields again peak materials corresponding to A and B with variable proportions. While acidic pH favor the formation of peak A material more in cold than at room temperature the alkaline pH seems to disfavor such a phenomenon. This tendency of aggregation of formyl peptides has been noticed in earlier studies on formyl polymyxins[3] and gramicidin A[4]. It seems likely that the aggregation of formyl peptides is at least partly due to their formyl group since deformylated F_1GS and formyl polymyxins do not exhibit such a phenomenon.

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