Gramicidin S Synthetase: Variations in the Activities of the Light and Heavy Enzymes with Growth of Culture of Bacillus brevis

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Gramicidin S is the cyclic decapeptide

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\text{D-Phe-L-Pro-L-Val-L-Orn-L-Leu} \quad \text{L-Leu-L-Orn-L-Val-L-Pro-D-Phe}
\]

which is synthesised by Bacillus brevis A.T.C.C. 9999 (Laland & Zimmer, 1973). It is synthesised by the enzyme gramicidin S synthetase, which activates and forms thioester bonds with each amino acid before peptide-bond formation (Gevers et al., 1969). Gramicidin S synthetase consists of two enzymes; the smaller, of mol. wt. 100000 (Yamada & Kurahashi, 1969), activates and forms a thioester bond with D-phenylalanine (Itoh et al., 1968); the second and larger enzyme has mol. wt. 280000 (Kleinkauf et al., 1969), and activates and forms a thioester bond with L-proline, L-valine, L-ornithine and L-leucine (Itoh et al., 1968). Pentapeptide units (D-Phe-L-Pro-L-Val-L-Orn-L-Leu) are formed, and two pentapeptides, probably synthesized by the same enzyme molecule, dimerize in a head-to-tail manner to form the cyclic decapeptide (Stoll et al., 1970).

Gramicidin S synthetase may be assayed in at least two different ways. The first is by measuring the synthesis of gramicidin S; this requires the presence of both large and small enzyme components. The second assay involves the measurement of an ATP-PP\(_i\) exchange in the presence of D-phenylalanine (which is a reaction given by the light enzyme and not by the heavy enzyme), or in the presence of L-ornithine (which is a reaction given by the heavy enzyme but not by the light enzyme).

Early experiments showed that cell-free extracts of Bacillus brevis could only synthesise gramicidin S during the late exponential phase of growth and that the activity of synthetase declined as the cells moved into the stationary phase (Laland & Zimmer, 1973). In order to begin a study of factors controlling the onset of gramicidin S production, experiments were undertaken to find out whether the activities of both light and heavy components of gramicidin S synthetase increased concomitantly with the ability to synthesize gramicidin S.

Bacillus brevis A.T.C.C. 9999 was grown in a 10-litre New Brunswick Laboratory Fermenter at 37°C as described previously (Bredesen et al., 1968). At various times, culture samples of 500ml and 10ml were withdrawn and rapidly cooled. The 10ml samples were used for determination of the total gramicidin S in the culture, by a modification of the method described by Eikhom et al. (1963). Cells from the 500ml samples were harvested and cell-free extracts prepared by treatment of the cells with lysozyme and deoxyribonuclease. Cell debris was removed by centrifuging for 20min at 11000gav. The supernatants were dialysed overnight against 0.05M-potassium phosphate buffer, pH7.6, containing 0.25mM-EDTA, 1mM-dithiothreitol and 20%\(v/v\) glycerol, and samples were assayed for gramicidin S synthetase activity as described previously (Freyshov et al., 1970). The light and heavy enzymes of gramicidin S synthetase were assayed by measuring ATP-PP\(_i\) exchange reactions in the presence of D-phenylalanine or L-ornithine by a method similar to that described by Simlot & Pfaender (1973). Controls, in which amino acid was omitted, were set up.

Fig. 1 shows that the gramicidin S synthetase activity of the crude extracts increased when they were prepared from samples taken during the exponential phase of growth. This increase in activity was accompanied by an increase in the activities characteristic of both the light and heavy components. This result is a direct confirmation of a result inferred by Kambe et al. (1974). The onset of gramicidin S synthesis in Bacillus brevis is dependent on the simultaneous appearance of both components.
Fig. 1. Gramicidin S synthetase activities at various times of culture of Bacillus brevis

Cells were harvested at various times of culture and cell-free extracts were prepared and dialysed overnight. The cell-free extracts were assayed for their ability to synthesize gramicidin S (○), for D-phenylalanine-dependent ATP-PP\textsubscript{i} exchange reaction (△), and for L-ornithine-dependent ATP-PP\textsubscript{i} exchange reaction (■). The gramicidin S content of the culture is also shown (▲). The growth of Bacillus brevis (●) was measured as $E_{650}$ after diluting samples of the culture ninefold.

Fig. 1 also shows that as the cells moved into the stationary phase, the ability of the cell-free extracts to synthesize gramicidin S declined. It is possible that this decline represents an artifact resulting from proteolytic activity in extracts prepared from cells in stationary-phase culture. This seems unlikely, since the gramicidin S content of the culture, which was measured directly, without preparation of a cell-free extract, was found to increase at the time of synthetase activity (Fig. 1), but soon reached a value which remained constant. This implies that there was a loss of synthetase activity in vivo, and it appears that the pattern of enzyme activities in the extracts reflect the situation in vivo. Fig. 1 shows that the loss of gramicidin S synthetase activity was associated with the almost complete loss of reactions of both the light and heavy enzymes. These sudden falls in activity, which are almost as rapid as the appearance of these enzymes, may be explained if the light and heavy enzymes have a high rate of turnover in vivo, so that switch-off of synthesis of these proteins results in a rapid loss of activity and cessation of synthesis of gramicidin S.
Use of Deuteriated Carrier plus Homologous Internal Standard in the Estimation of Prostaglandin \( \text{F}_2 \alpha \) by Gas Chromatography–Mass Spectrometry

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The use of a deuterium analogue for the measurement of prostaglandins by gas chromatography–mass spectrometry (g.l.c.—m.s.) by the use of multiple-ion monitoring was first reported by Samuelsson et al. (1970). The technique has since been used for the determination of a wide variety of drugs and metabolites. By using the 3,3,4,4-\( d_4 \) analogue of prostaglandin \( \text{F}_2 \alpha \), Grein et al. (1973) determined 400pg of \( \text{F}_2 \alpha \) with a standard deviation of ±3.7%.

Many workers favour an unlabelled homologue of the compound to be measured to act as an internal standard; this is chosen to give an identical ion so that single-ion monitoring can be used. By this means, Draffan et al. (1973) were able to quantify 20ng of amylobarbitone per ml of plasma, with a standard deviation of 7%.

Lee & Millard (1975) introduced the concept of using a deuterium-labelled version of the compound under measurement solely to act as a carrier, and, additionally, a homologue to act as the internal standard, so that the more sensitive single-ion-monitoring technique could be used. It was possible to quantify 40pg of a barbiturate with a coefficient of variation of 11%.

In the present work, a 100-fold excess of 3,3,4,4-\( d_4 \) prostaglandin \( \text{F}_2 \alpha \) was added as carrier for prostaglandin \( \text{F}_2 \alpha \). A synthetic analogue in which the side chain from C-16 onwards has been modified, was added also in 100-fold excess to act as the internal standard. The mixture was methylated with diazomethane and the trimethylsilyl derivatives formed. On a column of SE 30 at 260°C, the derivatives of prostaglandin \( \text{F}_2 \alpha \) and the analogue were well separated. Both compounds yield an intense ion at \( m/e \) 423 as follows:

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\text{Me}_3\text{SiO} + \text{OSiMe}_3
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\( m/e \) 423