STARTING POINT AND DIRECTION OF BIOSYNTHESIS OF GRAMICIDIN S

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Received 19 April 1969

1. Introduction

The biosynthesis of gramicidin S consists in joining five different amino acids, each occurring twice, in the cyclic structure:

D-Phe–L-Pro–L-Val–L-Orn–L-Leu
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L-Leu–L-Orn–L-Val–L-Pro–D-Phe

The enzymes responsible for gramicidin S synthesis have been purified to a considerable degree [1–4]. The mechanism of synthesis has also been the subject of study [2–5], but it is not known whether or not the synthesis starts with one particular amino acid, and if so, in which direction the synthesis proceeds. Here we wish to report experiments which are consistent with the view that the synthesis starts with phenylalanine and that the sequence of addition of amino acids is Phe→Pro→Val→Orn.

2. Materials and methods

2.1. *Gramicidin S synthetase*

Fraction 5 as prepared by Bredesen et al. [1] from *Bacillus brevis* ATCC 9999 was used as the source of gramicidin S synthetase.

2.2. *Incubation mixture*

The incubation mixture contained per ml: 0.1 ml of the enzyme solution (0.1 mg protein), triethanolamine hydrochloride (100 μmoles, pH adjusted to 7.6 with KOH), magnesium acetate (10 μmoles), ATP (2.5 μmoles), egg albumin (1 mg) and the appropriate amino acids (see tables 1 and 2).

2.3. *Isolation of gramicidin S and protein from the incubation mixture*

The method described previously [1] was used. Isolation of the protein fraction involved extraction with 5% (w/v) trichloroacetic acid containing 0.25% sodium tungstate, 2% (w/v) Na_2SO_4 solution and ethanol – 0.2 N HCl (9:1, v/v) to extract gramicidin S. The labelled gramicidin S was purified before counting by chromatography on thin layer silica plates in ethyl acetate-pyridine-acetic acid-water (60:20:6:11, v/v/v/v). Radioactivity was located by radioautography [6].

2.4. *Radioactive amino acids and measurement of radioactivity*

Uniformly labelled L-amino acids were purchased from New England Nuclear Corp. (Boston, Mass., USA). Radioactivity was measured in a Frieseke and Hoepfner windowless flow counter.

3. Results

The conclusions reached in the present work are based on pulse-chase experiments. In the pulse period, the incubation mixtures contained in each experiment only one labelled amino acid and were always deficient in some of the five constituent amino acids (from one to four in the different experiment) in gramicidin S. Hence only enzyme bound intermediates [5] and no gramicidin S could be synthesized during the pulse period. In all experiments the pulse period was terminated by immersing the incubation mixture

Table 1
Pulse-chase experiments using a single amino acid during the pulse period.

Amino acid present during pulse period	Increase in radioactivity in gramicidin S during the first 2 min of the chase period (counts/min)	Constant increment in radioactivity in gramicidin S per 2 min during chase period (counts/min)	Increase in radioactivity in gramicidin S as a result of the pulse (counts/min)	Decrease in radioactivity in protein fraction during the first 2 min of the chase period (counts/min)
[^{14}C]-Phe	5800	1800	4000	4400
[^{14}C]-Pro	1000	800	200	810
[^{14}C]-Val	600	700	−100	300
[^{14}C]-Orn	800	600	200	550
[^{14}C]-Leu	1000	800	200	700

In each experiment 0.01 μmole/ml of the unlabelled amino acid was added to the incubation mixture which was preincubated for 2 min at 37°. 4.0 μC of the corresponding [^{14}C]-labelled amino acid was then added to yield a specific activity of 50 C/mole. After 1 min at 37° (pulse period), the mixture was immersed in ice water for 10 min and a sample withdrawn for the estimation of radioactivity in gramicidin S and protein. To the reaction mixture was added appropriate amounts of all five amino acids present in gramicidin S. The final concentration of each amino acid was 0.5 μmole/ml and the final specific activity of the labelled amino acid was 1 C/mole. The mixture was then incubated at 37° and samples (1 ml) withdrawn during the chase period after 2, 4 and 6 min for the determination of radioactivity in gramicidin S and protein.

Table 2
Pulse-chase experiments using two, three and four amino acids during pulse period.

Amino acids present during pulse period	Increase in radioactivity in gramicidin S during the first 2 min of the chase period (counts/min)	Constant increment in radioactivity in gramicidin S per 2 min during chase period (counts/min)	Increase in radioactivity in gramicidin S as a result of the pulse (counts/min)	Decrease in radioactivity in protein fraction during the first 2 min of the chase period (counts/min)
[^{12}C]-Phe + [^{14}C]-Pro	2500	1500	1000	−250
[^{12}C]-Phe + [^{14}C]-Leu	1400	1300	100	300
[^{12}C]-Phe + [^{12}C]-Pro + [^{14}C]-Val	2500	1800	700	1100
[^{12}C]-Phe + [^{12}C]-Pro +[^{12}C]-Val + [^{14}C]-Orn	3200	2100	1100	1800

In each experiment 0.01 μmole/ml of the required unlabelled amino acids was added to the incubation mixture which was then preincubated for 2 min at 37°. 4.0 μC of the corresponding amino acid was then added to yield a specific activity of 50 C/mole in the incubation mixture. After 1 min at 37° (pulse period), the mixture was immersed in ice-water for 10 min and a sample withdrawn for the estimation of any radioactivity in gramicidin S and protein. To the reaction mixture was then added appropriate amounts of all five amino acids present in gramicidin S. The final concentration of each amino acid was 0.5 μmole/ml and the specific activity of the labelled amino acid was 1 C/mole. The mixture was incubated at 37° and samples (1 ml) withdrawn during the chase period after 2, 4 and 6 min for estimation of radioactivity in gramicidin S and protein.

in an ice-bath. This has been shown to decrease the incorporation to a negligible rate [5]. After dilution of the remaining labelled amino acid and addition of those amino acids in gramicidin S which were not present during the pulse period, the chase period was initiated by raising the temperature quickly to 37°. The incorporation of label into gramicidin S during the initial chase period was determined and compared with the decrease in the enzyme bound radioactivity.

In the first series of experiments, labelled phenylalanine, proline, valine, ornithine or leucine respectively was present during the pulse period (see table 1). It is seen from table 1 that the increase in radioactivity of gramicidin S during the initial 2 min chase period is substantial and that it was about the same as the decrease in the radioactivity in the protein fraction. The time course of the incorporation of radioactivity into gramicidin S is shown in fig. 1. The incorporation of radioactivity into gramicidin S during the first 2 min of the chase period increased from zero to 5800 counts/min. Then the rate (1800 counts/min per 2 min) became constant. The value of 4000 counts/min given in table 1 was obtained by subtracting the above values and represents the incorporation into gramicidin S as a result of the pulse.

In the experiments where the incubation mixture contained labelled proline, valine, ornithine or leucine during the pulse period, the increase in radioactivity in gramicidin S during the initial 2 min chase period (see table 1) was only insignificantly higher than the constant increment per 2 min chase period.

The above experiments demonstrate that phenylalanine occupies a special position in the biosynthesis of gramicidin S and suggest that it may be the starting point of synthesis. If this is the case and the synthesis proceeds in the direction Phe→Pro, then significant radioactivity should appear in the gramicidin S fraction during the chase period in an experiment where [^{12}C]-phenylalanine and [^{14}C]-proline only were present during the pulse period. This is seen to be the case (table 2). This is in contrast to the experiment when [^{14}C]-proline only was present during the pulse period, where negligible activity appeared in the gramicidin S fraction during the initial chase period (see table 1). Furthermore, if the direction of synthesis is Phe→Pro, then negligible radioactivity should appear in the gramicidin S fraction during the chase period when [^{12}C]-phenylalanine and [^{14}C]-leucine were present during the pulse period. It is also seen from table 2 that this is the case. In other experiments (not shown in table 2) where the incubation mixture contained either [^{12}C]-proline and [^{14}C]-valine or [^{12}C]-ornithine and [^{14}C]-leucine during the pulse period, negligible activity appeared in the gramicidin S fraction as a result of the pulse period.

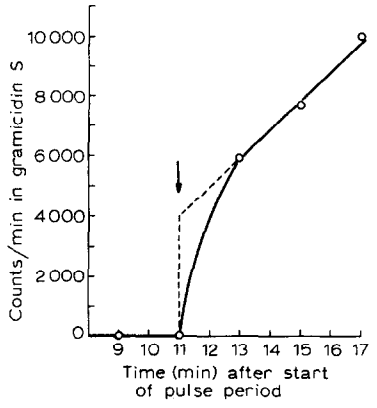

Fig. 1. Time course of incorporation of L-[^{14}C]-phenylalanine into gramicidin S in a pulse-chase experiment. For details see table 1. Chase period initiated at the point of arrow.

When the incubation mixture contained either [^{12}C]-phenylalanine, [^{12}C]-proline and [^{14}C]-valine or [^{12}C]-phenylalanine, [^{12}C]-proline, [^{12}C]-valine and [^{14}C]-ornithine during the pulse period, substantial activity appeared in the gramicidin S fraction during the chase period (see table 2). There was also a concomitant decrease in the radioactivity of the protein fraction. The results presented are consistent with the view that the synthesis starts with phenylalanine and that the sequence of addition of amino acids is Phe→Pro→Val→Orn.

4. Comments

In the experiments where [^{12}C]-phenylalanine and [^{14}C]-proline were used (table 2), there was no significant change in the radioactivity in the protein

fraction during the chase period, but a significant increase in the gramicidin S fraction. In this experiment the acidic ethanolic extract of the protein (see methods) obtained at the end of the pulse period, contained substantial amounts of radioactivity (3500 counts/min) in a substance which had chromatographic properties similar to that of the dipeptide Phe→Pro. In this particular experiment, the presumed protein bound intermediate for some reason seemed to be much less tightly bound or more easily split off from protein than in the other experiments where no intermediate was extractable with ethanol-HCl.

It appears from table 1 that the constant rate of gramicidin S synthesis during the chase period is lower wehn the incubation mixture did not contain phenylalanine during the pulse period. Previous experiments using a complete incubation mixture have shown [2] that all amino acids in gramicidin S are incorporated to the same extent. The present finding suggests that incubation of gramicidin S synthetase for a short period in the absence of phenylalanine reduces the subsequent rate of synthesis, e.g. by partly inactivating the enzyme system.

The present results indicate, as already suggested, that the syntehsis starts with phenylalanine and that the sequence of addition of amino acids is Phe→Pro →Val→Orn. Most probably leucine is then added to the C-terminal end. A different approach is required to settle this problem.

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