THE PRESENCE OF PROTEIN BOUND INTERMEDIATES IN THE BIOSYNTHESIS OF GRAMICIDIN S

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1. Introduction

Experiments with cell-free extracts from *Bacillus brevis* have established that the polypeptide gramicidin S is synthesized by a mechanism which is different from that of proteins [1]. When studying the mechanism of biosynthesis, the detection of possible intermediates is of obvious importance. Previous results [2] suggested that protein-bound intermediates may exist in the biosynthesis of gramicidin S. In the present work strong evidence for the presence of protein-bound intermediates is presented.

2. Methods and materials

2.1. *Preparation of a 40% $(NH_4)_2SO_4$ fraction as the source of the gramicidin S synthesizing activity*

The *B. brevis* strain ATCC 9999 was used and the 40% $(NH_4)_2SO_4$ fraction prepared from the cell-free extract as described in a previous paper [1]. The 40% $(NH_4)_2SO_4$ precipitate was dissolved in 0.02 M potassium phosphate buffer (pH 7.5) containing GSH (10 μmoles/ml) and glycerol (20%, v/v), and dialyzed against the same buffer at +3° for 12 hr. The solution contained in most experiments 20–30 mg protein per ml.

The 40% $(NH_4)_2SO_4$ fraction which was used as the source of gramicidin S synthesizing activity, incorporated into gramicidin S the different constituent amino acids to the same extent.

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2.2. *Incubation mixture*

All mixtures contained per ml: 0.5 ml of enzyme solution (see above), triethanolamine hydrochloride (100 μmoles, pH adjusted to 7.6 with KOH), KCl (5 μmoles), mercaptoethanol (6 μmoles), magnesium acetate (50 μmoles), ATP (2.5 μmoles) and L-phenylalanine, L-leucine, L-ornithine, L-proline and L-valine, one of which was labelled. The concentrations of the amino acids and the amount of radioactivity used are given in each experiment.

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

A sample of the incubation mixture (0.1 ml) was mixed at 0° with 0.1 ml of ice-cold 10% (w/v) trichloroacetic acid containing 0.5% of sodium tungstate and 0.1 M of the [^{12}C] L-form of the labelled amino acid used. The mixture was added 25 μg of gramicidin S and the precipitate washed twice at room temperature with 5% (w/v) trichloroacetic acid containing 0.25% sodium tungstate, 1 ml each time, and once with an aqueous 2% (w/v) Na_2SO_4 solution (1 ml). Gramicidin S was then extracted with 1 ml of ethanol-0.2 N HCl (9:1, v/v) at room temperature for 3 hr. The residue was washed with ethanol-0.2 N HCl mixture, 0.5 ml each time and the combined extract and washings used for the estimation of radioactivity in gramicidin S. The residue after gramicidin S extraction was washed with ethanol (96%, w/v) and dissolved in 0.1 N NaOH (0.25 ml) and used for counting and estimation of protein [3]. The purity of the labelled gramicidin S fraction was checked by chromatography on thin layer silica plates in ethyl acetate-pyridine-acetic acid-water (60:20:6:11, v/v) [4] and

radioactivity located by radioautography. In all cases only one labelled spot with R_F similar to that of gramicidin S was found.

2.4. *Radioactive amino acids and measurement of radioactivity*

L-[U-^{14}C]leucine (251 mC/mmole and L-[U-^{14}C]-valine (190 mC/mmole) were purchased from New England Corp., Boston, Mass., USA. Radioactivity was measured at infinite thinness in a Frieseke and Hoepfner windowless flow counter.

3. Results

In order to demonstrate the presence of protein bound intermediates, two types of experiments have been carried out. These are pulse-chase experiments and experiments where the effect of the composition of the incubation mixture on the conversion of the protein bound intermediates into gramicidin S was studied.

3.1. *Pulse-chase experiment*

The result of a pulse-chase experiment using [^{14}C]-leucine is seen in fig. 1. The pulse period was interrupted by cooling the incubation mixture to 0°. It is seen from fig. 1 that the rate of gramicidin S synthesis at this temperature is negligible. The chase-period was initiated by raising the temperature to 37° at a 20 X dilution of labelled leucine. It appears from fig. 1 that upon reincubation there is an initial rapid increase in the radioactivity of gramicidin S followed by a slower linear increase. At the same time there is a rapid decrease in the radioactivity of the protein fraction. The rapid changes in the gramicidin S and protein fractions were estimated by extrapolating the two curves back to the beginning of the chase period. It is noteworthy that the decrease in the radioactivity of the protein fraction (approx. 900 counts/min) roughly equals the increase in the gramicidin S fraction (approx. 1000 counts/min). Similar results were obtained in pulse-chase experiments with labelled proline, ornithine and valine. In some of these experiments the decrease in the protein fraction would be somewhat higher than the increase in the gramicidin S fraction. In the case of phenylalanine, there was also a decrease in the protein fraction with a simultaneous increase in the gramicidin S fraction. However, the decrease in the protein fraction was roughly twice the increase in the gramicidin S fraction.

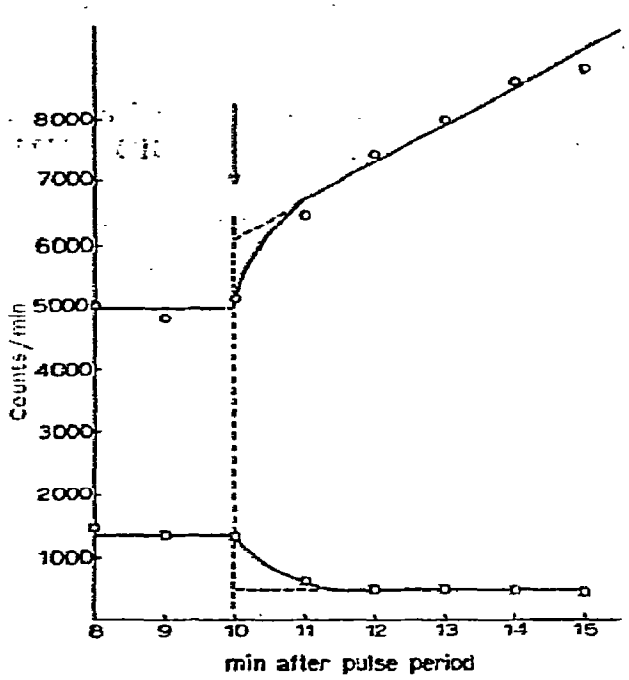

Fig. 1. *Pulse-chase experiments with 40% $(NH_4)_2SO_4$ fraction using ^{14}C leucine.* An incubation mixture (1 ml) containing 0.05 μmole of each amino acid was preincubated at 37° for 2 min before the addition of the labelled amino acid (2.5 μC). 30 sec later the mixture was placed in icewater. Samples were withdrawn after 8, 9 and 10 min. After a period of 10 min in icewater, at a point of the arrow, the tube with the remaining incubation mixture was added L-[^{12}C]leucine at a 20 X dilution of the labelled amino acid and the mixture incubated at 37°. Samples were then withdrawn at intervals. o—o and □—□, radioactivity in the gramicidin S and protein fraction, respectively.

The results obtained represent strong evidence for the existence of protein-bound intermediates in the biosynthesis of gramicidin S.

3.2. *Factors influencing the conversion of the protein bound intermediates into gramicidin S*

Gel filtration at +3° of an incubation mixture containing [^{14}C]valine (see table 1) yielded a labelled protein fraction which was free from amino acids and other low molecular weight substances. It contained,

Table 1

The effect of the composition of the incubation mixture on the conversion of the protein-bound radioactivity into gramicidin S.

Treatment of labelled protein	Radioactivity (counts/min)	
	Gramicidin S	Protein
Not reincubated (control)	600	1680
Reincubated with ATP, Phe, Pro, Val, Orn, Leu	1500	500
Reincubated with ATP	640	1180
Reincubated with Phe, Pro, Val, Orn, Leu	660	1400

An incubation mixture (4 ml) containing 0.01 μmole of each amino acid was kept at 37° for 2 min before addition of [^{14}C]-valine (2.5 μC). One minute after the addition of the labelled amino acid the incubation mixture was cooled in ice-water. The mixture was then fractionated at +3° on a column (16 cm × 1.6 cm, height × diam.) of a Sephadex G-50 course grade. The column was equilibrated with a 0.1 M-triethanolamine hydrochloride buffer adjusted to pH 7.6 with KOH containing mercaptoethanol (6 μmoles/ml), KCl (5 μmoles/ml), glycerol (10%, w/v) and 0.05 M magnesium acetate and eluted with the same solution at a rate of 1 ml/min. When the void volume had emerged, the next 4 ml, containing labelled proteins, were collected and used as the enzyme source in the reincubation experiments. Part of the same fraction was used for the determination of radioactivity in protein and gramicidin S. The remaining of the labelled fraction was used immediately for the reincubation experiment. 1 ml of enzyme solution was added 2.5 μmole of ATP and, when desired, 0.05 μmole of the individual amino acids and incubated for 2 min at 37° and the radioactivity in protein and gramicidin S then determined.

however, a small proportion of labelled gramicidin S, probably as a result of adsorption. The labelled protein fraction was reincubated at 37° in order to study the conversion of a possible protein-bound intermediate into gramicidin S. It is seen that when the labelled protein is reincubated with ATP and the five amino acids, the amount of label which disappears from the protein fraction roughly equals the increase in the gramicidin S fraction. Furthermore, reincubation in the absence of ATP or the five amino acids results in no significant increase in the radioactivity of gramicidin S. There is, however, a significant decrease in radioactivity in the protein fraction. The results in table 1 lead to the interesting conclusion that both ATP and the five amino acids in gramicidin S are required for the label to pass from the protein to gramicidin S.

When the protein was labelled with leucine, proline or ornithine, reincubation in the presence of ATP and the five amino acids also resulted in a decrease in the protein and a roughly equal increase in the gramicidin S fraction. In the case of phenylalanine the decrease in the radioactivity of the protein fraction was considerably higher than the increase in the gramicidin S fraction.

These results, thus, lend further support to the view that protein-bound intermediates exist.

4. Comments

The pulse-chase experiments and the reincubation experiments using protein labelled with either one of the five amino acids in gramicidin S, indicate that protein-bound intermediates exist in the biosynthesis of gramicidin S. The finding that the conversion of the protein-bound intermediates into gramicidin S after incubation requires the presence of ATP and the five constituent amino acids, shows that it is a synthetic process and not merely a liberation of preformed and still bound gramicidin S. The bond between the protein and the intermediate is stable to trichloroacetic acid at room temperature. The present findings are important for the further studies on the mechanism of the biosynthesis of gramicidin S. Attempts are being made to isolate protein-bound intermediates.

Fujikawa, Suzuki and Kurahashi [5] presented indirect evidence for the presence of intermediates bound to the enzyme in the biosynthesis of the polypeptide tyrocidin. The present result is to our knowledge the first direct evidence for the presence of protein-bound intermediates in the biosynthesis of bacterial polypeptides.

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