THE NATURE OF THE ENZYME BOUND INTERMEDIATES IN GRAMICIDIN S BIOSYNTHESIS

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1. Introduction

The biosynthesis of gramicidin S consists in joining 5 different amino acids, each occurring twice, in the cyclic structure:

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

Previous work [1, 2] indicated that during synthesis, intermediates are covalently bound to gramicidin S synthetase. Furthermore, the synthesis starts with phenylalanine and the sequence of addition of amino acids is Phe–Pro–Val–Orn [2]. When the present work was completed, a paper by Gevers, Kleinkauf and Lipmann [3] appeared which indicated that all five amino acids and the intermediate peptides D–Phe–L–Pro, D–Phe–L–Pro–L–Val, D–Phe–L–Pro–L–Val–L–Orn, and D–Phe–L–Pro–L–Val–L–Orn–L–Leu are bound covalently to the synthetase, most probably through thioester linkages. This work presents additional evidence for this view. The present results also show that there is a marked difference in the stability of the linkages of ornithine and D–Phe–L–Pro to the protein towards ethanol-HCl compared to that of the other covalently bound intermediates. Using a different method to that of Gevers et al. [3] no evidence for peptides longer than the pentapeptide was found. This finding supports the view that gramicidin S is formed by head to tail condensation of activated pentapeptides

2. Materials and methods

2.1. *Gramicidin S synthetase*

Fraction 5 was prepared as described by Bredesen et al. [4] from *Bacillus brevis* ATCC 9999 and was used as the source of gramicidin S synthetase.

2.2. *Incubation mixture*

The incubation mixture contained per ml: triethanolamine hydrochloride (100 μmoles, pH adjusted to 7.6 with KOH), KCl (5 μmoles), mercaptoethanol (6 μmoles), magnesium acetate (10 μmoles), ATP (2.5 μmoles), enzyme (0.1 mg), egg albumin (1 mg) and the appropriate amino acids (see tables 1 and 2 and fig. 1). All incubations were carried out at 37°C for 3 min.

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

The method previously described was used [2]. Isolation of the protein fraction involved precipitation with 10% trichloroacetic acid (w/v), extraction with 5% (w/v) trichloroacetic acid, 2% (w/v) Na_2SO_4 solution and with ethanol-0.2 N HCl (9:1, v/v) at room temperature to extract gramicidin S.

2.4. *Liberation of enzyme bound intermediates*

Enzyme bound intermediates were liberated by performic acid oxidation (a) or by treatment with KOH (b).

a. The protein (1 mg) isolated from the incubation mixture was dissolved in 0.25 ml 99% HCO_2H and 0.05 ml ethanol. The mixture was cooled to −10°C and 0.1 ml performic acid added [5]. The mixture was left at this temperature for 2.5 hr diluted with

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water and freeze-dried. The dried material was extracted with 96% ethanol to remove liberated amino acids and peptides. The protein fraction was dried and submitted to two further oxidations with performic acid. All ethanol extracts were combined.

b. The protein (1 mg) was dissolved in 0.1 N KOH and kept at 20°C for 1 hr. 0.5 N $HClO_4$ was added to adjust the mixture to pH 3.0 and the supernatant was concentrated to dryness in a desiccator. The solid was extracted with ethanol to remove liberated amino acids and peptides.

2.5. *Thin-layer chromatography*

Two dimensional chromatography was carried out on thin-layer silica plates (medium H according to Stahl, E.Merck AG, Darmstadt, Germany). Ethyl acetate-pyridine-acetic acid-water (60:20:6:11, by vol) were used in the first dimension and butan-1-ol-pyridine-acetic acid-water (5:3:1:1, by vol) in the second dimension.

2.6. *Radioactive amino acids and measurement of radioactivity*

Uniformly labelled L-amino acids were purchased from New England Corp., Boston, Mass., U.S.A. Radioactivity was measured at infinite thinness in a Frieseke and Hoepfner windowless flow counter. Radioactivity on thin-layer plates was located by radioautography using Ilford Industrial G film.

3. Results

The purpose of the present work was to determine the type of covalent bonds between the intermediates and gramicidin S synthetase and the nature of the intermediates.

Table 1
Liberation of protein bound radioactivity with performic acid and KOH.

	% Radioactivity removed from protein			% Radioactivity remaining in protein
	No of treatments			
	1	2	3	
Performic oxidation	75	19	4.5	1.5
0.1 N KOH	80	18		2

The incubation mixture (see **Materials and methods**) contained per ml, 0.05 μmole of L-phenylalanine, L-proline, L-valine, L-ornithine and L-leucine each with a specific activity of 50 Ci/mole.

Table 2
Determination of protein bound intermediates after incubation of gramicidin S synthetase with ^{14}C-phenylalanine and combinations of amino acids which do not permit gramicidin S synthesis.

Amino acids present in incubation mixture	Labelled substances liberated from protein with performic acid	Labelled substances in ethanol-0.2 N HCl extract of the protein
^{14}C–L–Phe, L–Pro, L–Val, L–Orn	^{14}C–Phe, ^{14}C–Phe–Pro–Val ^{14}C–Phe–Pro–Val–Orn (^{14}C–Phe–Pro–Val–Orn–Leu)	(^{14}C–Phe), A (G.S)
^{14}C–L–Phe, L–Pro, L–Val	^{14}C–Phe, ^{14}C–Phe–Pro–Val (^{14}C–Phe–Pro–Val–Orn)	(^{14}C–Phe), A
^{14}C–L–Phe, L–Pro	^{14}C–Phe, (^{14}C–Phe–Pro–Val)	(^{14}C–Phe), A
^{14}C–L–Phe, L–Orn, L–Leu	^{14}C–Phe	(^{14}C–Phe), (A)

Each incubation mixture contained per ml 0.05 μmole of ^{14}C–L–Phe with specific activity 50 Ci/mole and 0.05 μmole of each of the amino acids indicated in the table. All radioactive substances liberated from the protein fraction as well as those present in the ethanol-1.1 N HCl extract were determined by thin layer chromatography. A denotes ^{14}C–Phe–Pro diketopiperazine and G.S denotes gramicidin S. The substances in brackets were only present in trace amounts.

It appears from the experiment described in table 1, where the incubation mixture contained all of the five amino acids in gramicidin S in labelled form, that all protein bound radioactivity was liberated by performic acid oxidation or by treatment with 0.1 N KOH at 20°C. Prior to this treatment the protein had been exposed to trichloroacetic acid and ethanol-0.2 N HCl (see Methods). Hence the bound intermediates are stable to acid, but are removed from the protein by treatment with alkali or performic acid. This strongly suggests thioester bonds between the intermediates and gramicidin S synthetase, analogous to that found in fatty acid synthetase, [6] are involved.

In order to identify the nature of the enzyme bound intermediates, the substances liberated by performic acid oxidation after incubation of gramicidin S synthetase with the five labelled amino acids were submitted to two dimensional thin-layer chromatography after addition of unlabelled amino acids and the synthetic peptides D–Phe–L–Pro–Val, D–Phe–L–Pro–L–Val–L–Orn, D–Phe–L–Pro–L–Val–L–Orn–L–Leu.* It appears from fig. 1 that performic acid oxidation liberated three peptides which cochromatographed with the added tri, tetra and pentapeptides. Separate experiments using only one labelled amino acid in each incubation mixture confirmed the composition of the three peptides. Furthermore, labelled phenylalanine, proline, valine, and leucine were also liberated from the protein. Labelled ornithine was absent. However, thin-layer chromatography of the 0.2 N HCl-ethanol extract (used prior to performic oxidation, see Methods) of the protein revealed the presence of ornithine and D–Phe–L–Pro diketopiperazine. Thus, it appears that the dipeptide is split off the protein as the diketopiperazine derivative during treatment with 0.2 N HCl-ethanol and that its linkage to the protein is less stable under these conditions than are the linkages between the protein and the tri, tetra and pentapeptides. Similarly, the linkage between protein and ornithine is less stable to 0.2 N HCl-ethanol at room temperature than the linkages between the protein and phenylalanine, proline, valine and leucine. However, the dipeptide and ornithine are not liberated from the protein in the presence of cold trichloroacetic acid (see Methods). By analogy with the other amino

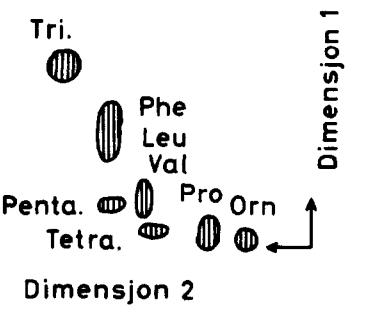

Fig. 1. Determination of labelled substances liberated from gramicidin S synthetase by performic oxidation after incubation with all five amino acids in labelled form. The incubation mixture contained 0.05 μmole of each amino acid with a specific activity of 50 Ci/mole. Unlabelled L–Phe, L–Pro, L–Val, L–Orn, and L–Leu as well as the peptides D-Phe–L–Pro–L–Val, D–Phe–L–Pro–L–Val–L–Orn' D–Phe–L–Pro–L–Val–L–Orn–L–Leu were added to the material libetared from the protein by exhaustive performic acid oxidation before two-dimensional chromatography (see Methods). The plate was developed by radioautography and ninhydrin. The positions of the reference spots (ninhydrin) are indicated. All spots except ornithine were labelled.

acids and peptides, it seems reasonable to assume that ornithine and D–Phe–L–Pro are bound to the enzyme through thioester linkages. The absence of peptides larger than the pentapeptide supports the view that gramicidin S is formed by the head to tail condensation of two activated pentapeptides.

In order to decide whether a molecule of gramicidin S synthetase contains several growing peptide chains, the following experiment was carried out. ^{14}C–L–Phe was incubated together with L–Pro, L–Val, and L–Orn, and gramicidin S synthetase. Because of the absence of L–Leu, the growth of the peptide chain stops at the stage of the tetrapeptide. The existence of smaller peptides bound to the enzyme would indicate that each enzyme contained several growing peptide chains. The result is reported in table 2. It appears that ^{14}C–Phe, ^{14}C–D–Phe–L–Pro–L–Val and ^{14}C–D–Phe–L–Pro–L–Val–D–Orn were liberated from the enzyme by performic acid. The tri- and the tetrapeptide were present in about equal amounts. In addition, the ethanol-0.2 N HCl extract contained ^{14}C–D–Phe–L–Pro diketopiperazine. Hence the results support the view that each enzyme contains several growing peptide chains. Initiation of more than one chain occurred also when the incubation mixture contained ^{14}C–L–Phe,

* The synthetic peptides were a gift to Mr. H.Holm from Osaka City Medical Center, Protein and Peptide Laboratory, Osaka.

L–Pro, and L–Val since the major peptides liberated were ^{14}C–D–Phe–L–Pro diketopiperazine and ^{14}C–D–Phe–L–Pro–L–Val (see table 2). As expected, the enzyme bound intermediates were ^{14}C–Phe and ^{14}C–Phe–Pro diketopiperazine when the incubation mixture contained ^{14}C–L–Phe and L–Pro. After incubation with ^{14}C–L–Phe, this amino acid was bound to the enzyme. Addition of L–Orn and L–Leu to ^{14}C–L–Phe gave, as expected, rise to no further intermediates.

It seen from table 2 that in every experiment the protein contained traces of a peptide (in brackets) with one amino acid more than those present in the incubation mixture. For instance there were trace amounts of the pentapeptide and gramicidin S in the experiment where the incubation contained only four amino acids. The most likely explanation is that traces of activated amino acids bound to the synthetase, at the time when cells are harvested, remain bound throughout the purification procedure.

4. Comments

As previously shown [7, 8], gramicidin S synthetase consists of two enzymes, enzyme I and enzyme II. Enzyme I which is the larger, activates L-proline, L-valine, L-ornithine, L-leucine. Enzyme II activates and racemizes L-phenylalanine. On the basis of the present results and those of Gevers et al. [3] the following mechanism for gramicidin S synthesis emerges:

At the start of synthesis, the five amino acids become activated through the formation of aminoacyl adenylates [7, 9]. Each of the five aminoacyl groups is then transferred to a specific thiol group on the enzyme. The D-phenylalanyl group in a thioester linkage is then transferred to the amino group of an adjacent prolyl group also linked through a thioester bond to the enzyme. The D-phenylalanylprolyl group is then transferred to the amino group of the valyl group and so on. The empty thiol groups are filled up with new amino acyl groups and another peptide chain is initiated before the first one is completed. In this model the sequence of the amino acids in gramicidin S is determined by the order of the thioester linked amino acids on the surface of the enzyme complex. Two pentapeptides linked through its carboxyls to thiol groups then condense head to tail to give gramicidin S.

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