THE PRESENCE AND POSSIBLE ROLE OF PHOSPHOPANTOTHENIC ACID IN GRAMICIDIN S SYNTHETASE

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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 .

Previous work on the biosynthesis of gramicidin S has shown that intermediates are covalently bound to the enzyme [1, 2] and that they can be split off by treatment with alkali [1], performic acid [1, 3], mercury salts or hydroxylamine [4]. Furthermore, it has been demonstrated that the synthesis starts with phenylalanine and proceeds in the direction Phe → Pro → Val → Orn → Leu [5]. In agreement with this finding it was shown independently by Gevers, Kleinkauf and Lipmann [4] and Frøyshov, Zimmer and Laland [3] that di-, tri-, tetra-, and pentapeptides all having D-phenylalanine at the *N*-terminal end as well as all five amino acids in gramicidin S are bound to the enzyme through thioester linkages.

The possible presence of 4′-phosphopantetheine in gramicidin S synthetase occurred to us some time ago when evidence for the presence of acid stable and alkali labile enzyme intermediates was obtained [1, 2]. Preliminary analysis of a gramicidin S synthetase preparation, designated fraction 5 and described in a previous publication [6] had indicated the presence of pantothenic acid [7] *. Since the enzyme used was not pure enough to establish this finding with certainty, the final proof had to await the preparation of a pure enzyme.

The present work with a pure enzyme demonstrates that upon alkaline treatment of the two enzymes involved in gramicidin S synthesis, approximately one molecule of phosphopantothenic is liberated from one molecule of the larger enzyme. The detection of phosphopantothenic acid and thioester linked intermediates in gramicidin S synthetase makes the presence of phosphopantetheine, as in fatty acid synthetase [8], a probability. The growing peptide chain could during gramicidin S synthesis be attached through a thioester bond to the arm of phosphopantetheine. The function of the phosphopantetheine arm would then be to bring the growing peptide chain to the site of the subsequent activated amino acid (in a thioester linkage) on the surface of the enzyme.

2. Methods and materials

2.1. *Gramicidin S synthetase*

Fraction 5 prepared as described by Bredesen et al. [6] from *Bacillus brevis* ATCC 9999 was used as the source of gramicidin S synthetase.

2.2. *Assay for gramicidin S synthesizing activity*

The incubation mixture and the procedure for isolation of gramicidin S were as previously described [3].

* Microbiological analysis carried out by Dosent O.Braekkan, Vitaminlaboratoriet, Bergen, February 1969.

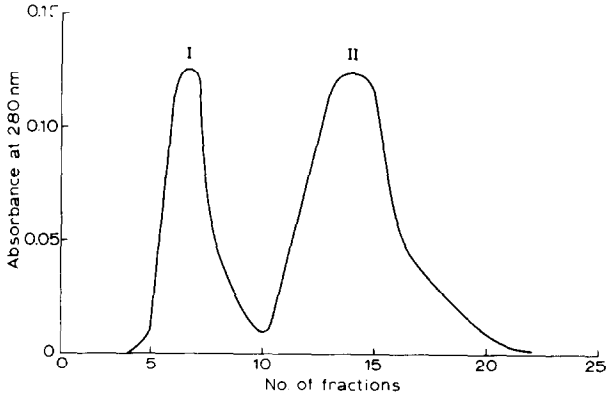

Fig. 1. Fractionation on Sephadex G-200. 8 mg protein of fraction 5 (see Methods and materials) was put on a column (2.5 X 34 cm) and eluted with a 0.05 M potassium phosphate buffer, pH 7.5, containing 1 mM dithiothreitol, 0.20 mM EDTA, and 20% glycerol. Fractions of 5 ml were collected.

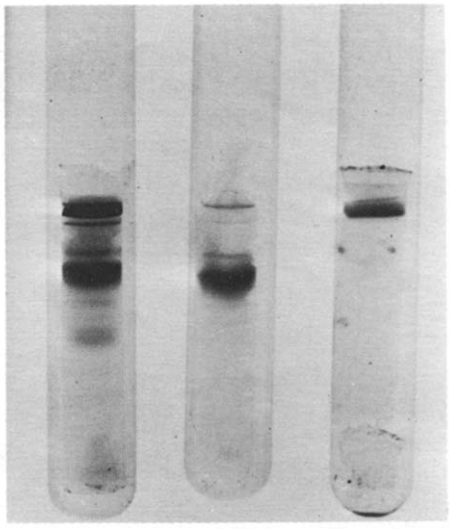

Fig. 2. Polyacrylamide disc electrophoresis of fraction 5, peak I, and peak II at pH 7.5 and 7.5% acrylamide: (A) Fraction 5, (B) Peak II, and (C) Peak I.

2.3. *Activation of amino acids*

The activation of the amino acids in gramicidin S was measured by the ATP-^{32}P-pyrophosphate exchange reaction according to the general procedure of Calendar and Berg [9].

2.4. *Polyacrylamide disc electrophoresis*

The Polyanalyst Apparatus of Buchler was used. Samples were run at pH 7.5 and 9.3 with gels containing 7.5 or 5% acrylamide.

2.5. *Release of pantothenic acid from the enzyme*

The procedure of Pugh and Wakil [10] was followed. The protein (approx. 0.25 mg) was hydrolyzed in 1 N KOH at 100° for 2 hr. After neutralization with 1 N HCl, tris-HCl buffer pH 8.0 was added to final concentration 0.05 M and the mixture was incubated with alkaline phosphatase (Worthington, 2.5 mg/ml) for 1 hr at 37°. At the end of the incubation, pH was adjusted to 4.5 with acetic acid, the precipitated protein removed by centrifugation, and the supernatant was assayed for pantothenic acid. Appropriate blanks were run simultaneously.

2.6. *Assay for pantothenic acid*

The test organism, *Lactobacillus plantarum* ATCC 8014, was maintained as stab cultures on Bacto Pantothenate Culture Agar. Subculturing and assay for pantothenic acid were performed using Bacto Pantothenate Medium USP according to the method of Difco [11] which is based on USP [12] description.

2.7. *Estimation of protein*

Protein was determined with the Folin-Ciocalteu reagent method of Lowry et al. [13] using bovine albumin as a standard.

2.8. *Radiochemicals and measurement of radioactivity*

Uniformly labelled ^{14}C-phenylalanine was purchased from New England Nuclear Corp., Boston, Mass., USA. ^{32}P-labelled inorganic pyrophosphate was obtained from The Radiochemical Centre, Amersham, England. Radioactive samples were counted in a Frieseke and Hoepfner windowless flow counter.

3. Results

In order to test for the presence of pantothenic acid in gramicidin S synthetase, fraction 5 (see

Table 1
Activation of amino acids by peak I and peak II (see fig. 1).

Amino acid	Cpm in ATP per 25 μg of protein	
	Peak I	Peak II
L-Phe	230	2100
D-Phe	–	1700
L-Pro	1590	55
L-Val	2420	80
L-Orn	2520	17
L-Leu	3340	70

Each incubation mixture contained in 1 ml: 25 μg of protein, triethanolamine buffer pH 7.6 (100 μmoles), KCl (5 μmoles), mercaptoethanol (6 μmoles), one amino acid as indicated (2 μmoles), ATP (2.5 μmoles), ^{32}P-labelled sodium pyrophosphate (2.5 μmoles, 0.03–0.07 μCi). The incubation was carried out for 5.5 min at 37°.

Materials and methods) was further purified by chromatography on a Sephadex G 200 Column (fig. 1). Two peaks were then obtained. Polyacrylamide disc electrophoresis of the peaks showed that peak I gave rise to one band only at two different pH values, and at different degree of crosslinking of the gels. Peak II gave one main band but was contaminated with small amounts of other proteins. Some of the results are shown in fig. 2. Neither peak I nor peak II were able to effect synthesis of gramicidin S alone, but when combined, incorporation of ^{14}C-phenylalanine into the decapeptide occurred. Peak I was found to activate specifically L-proline, L-valine, L-ornithine and L-leucine, and peak II D- and L-phenylalanine with virtually no overlapping of the two peaks (table 1). The finding that phenylalanine is activated by a separate enzyme is in agreement with that reported previously by other workers [15, 16]. In their experiments, however, there were more overlapping between the two activating enzymes.

Since it seemed possible that pantothenic acid was present as 4′-phosphopantetheine in gramicidin S synthetase as is the case in fatty acid synthetase, a similar method [10] for the liberation of pantothenic acid was used in the present case. The method involves treatment of the protein with alkali which will liberate phosphopantothenic acid from protein bound 4′-phosphopantetheine. Subsequent digestion with alkaline phosphatase releases pantothenic acid

Table 2
Estimation of pantothenic acid in peak I and peak II (for details see Methods and materials).

Preparation	Pantothenic acid** (ng)	Protein (μg)
Peak I	150	250
Peak I*	0	250
Peak II	0	250
Control	0	–

* Alkaline phosphate treatment omitted.
** Mean of results from two different preparations.

which is then assayed with a pantothenic acid requiring organism. The results are presented in table 2. Only peak I yielded pantothenic acid. It is further seen that when treatment with alkaline phosphatase was omitted, the sample did not support the growth of the organism. This suggests that, as expected, phosphopantothenic acid was present in the alkaline digest of the protein since the phosphate derivative is known not to support the growth of the test organism [10]. Assuming a molecular weight of 280,000 [14] for the enzyme in peak I, the result gives 0.8 moles of phosphopantothenic acid per molecule of enzyme.

4. Discussion

The present results demonstrate that phosphopantothenic acid is an integral part of gramicidin S synthetase. Of the two proteins (peak I and peak II) participating in gramicidin S synthesis, one molecule of phosphopantothenic acid is attached to the larger protein which activates proline, valine, ornithine and leucine and which contains all intermediate peptides bound through thioester linkages [15]. The protein which activates and racemized phenylalanine does not contain phosphopantothenic acid.

Although there is no definite proof that phosphopantothenic acid is present as phosphopantetheine in gramicidin S synthetase, it seems a very likely possibility. On this basis and in analogy with what has been suggested in fatty acid synthetase [8] we should like to suggest the following role of a phosphopantetheine arm in the biosynthesis of gramicidin S.

The activated thioester linked phenylalanyl group

[3, 4] located on the enzyme complex is transferred to the thiol group of the phosphopantetheine arm. By movement of the arm, the phenylalanyl group is brought close to the site of the activated thioester linked prolyl group on the surface of the enzyme. The phenylalanyl group is then transferred from the arm to the amino group of the activated prolyl group with the formation of a dipeptide. The thioester linked phenylalanylprolyl group is then transferred to the now free thiol group of the arm which then by movement brings the activated dipeptide to the next site which contains the thioester linked valyl group, and by transfer the formation of the tripeptide occurs. The tripeptidyl group is then transferred to the thiol group of the arm and by further movement the formation of the tetra- and the pentapeptide takes place in an analogous manner. Since the enzyme seems to contain only one molecule of phosphopantothenic acid, a mechanism as outlined above would be consistent with one growing chain per enzyme complex.

Previous experiments with gramicidin S synthetase using an incubation mixture lacking in the last amino acid (leucine), indicated that the protein contained the di-, tri- and tetrapeptides as intermediates [3]. This was taken as evidence that one enzyme contains several growing peptide chains [3] but would be difficult to reconcile with the presence of one phosphopantetheine arm. However, this may be a rather special case and not representative of what is happening in a complete incubation mixture. If leucine is lacking, the growth of the peptide chain would stop at the tetrapeptide stage. The arm could then move back and pick up another molecule of phenylalanine which had become activated in the meantime and initiate the growth of a second peptide chain, which would stop at the tripeptide stage. A third chain could become initiated in a similar manner which would then stop at the dipeptide stage. This then would explain why in the absence of leucine the growth of several peptide chains are intitiated.

When using ^{14}C-Phe as the labelled amino acid and comparing the amount of protein bound intermediates in an incubation mixture lacking in leucine and in an incubation where all five amino acids were present, it was found that the former contained considerably more of labelled intermediates [3, 4]. This difference is again explained if in the incomplete mixture gramicidin S synthetase contains several growing chains as compared to one only in the complete incubation mixture.

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