ON THE MECHANISM OF GRAMICIDIN S FORMATION FROM INTERMEDIATE PEPTIDES

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1. Introduction

Previous work [1] has shown that gramicidin S synthesis starts with phenylalanine and proceeds in the direction Phe → Pro → Val → Orn → Leu and that all intermediate peptides are covalently bound to the enzyme [2, 3]. Since the largest peptide found was always the pentapeptide, it was concluded that gramicidin S is formed by a head to tail condensation of two activated pentapeptides. The present work shows that once the larger (enzyme I) of the two enzymes participating in gramicidin S synthesis is charged with intermediate peptides, gramicidin S synthesis can take place in the absence of the smaller enzyme (enzyme II, phenylalanine activating and racemizing enzyme). Furthermore, our experiments strongly suggest that the condensation of the two pentapeptides is an intramolecular reaction i.e. that two pentapeptides linked to the same enzyme I molecule undergo head to tail condensation. On the basis of this finding, a scheme for the cyclisation reaction involving the 4′-phosphopantetheine arm is suggested.

2. Methods and materials

2.1. *Gramicidin S synthetase*

Fraction 5 prepared as described by Bredesen et al. [4] from *Bacillus brevis* ATCC 9999 was used in all experiments. The enzyme was concentrated by ammonium sulfate precipitation and dialyzed against 20 mM potassium phosphate, pH 7.5, containing 20% glycerol, 0.25 mM EDTA and 1 mM dithiothreitol (DTT).

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

The protein was precipitated and gramicidin S was determined as described previously [1]. Before counting, the protein residue was dissolved in formic acid.

2.3. *Estimation on free and protein bound ^{14}C labelled phenylalanine*

Free phenylalanine and protein bound phenylalanine liberated by oxidation with performic acid were estimated by the isotope dilution method. A known amount of ^{12}C-phenylalanine was added and the isolated amino acid recrystallized to constant specific activity.

2.4. *Thin-layer chromatography*

Medium H according to Stahl, E.Merck AG, Darmstadt, Germany, with ethyl acetate–pyridine–acetic acid–water (60:20:6:11 by vol.) as the solvent was used. If the R_f value of gramicidin S was not approximately 0.5, a small amount of water was added to the solvent to achieve this.

2.5. *ATP–^{32}P-pyrophosphate exchange*

This reaction was measured according to the general procedure of Calendar and Berg [5].

2.6. *Labelled substances and measurement of radioactivity*

4-^{14}C-D,L-Azetidine-2-carboxylic acid was purchased from Calatomic, Los Angles, U.S.A. U-^{14}C-L-Phenylalanine and U-^{14}C-L-leucine were obtained from New England Nuclear Corporation, Boston, Mass., U.S.A.

Radioactivity was measured in a liquid scintillation counter. Radioactivity on thin plate layer was located by radioautography.

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3. Results

3.1. *Incorporation of enzyme I bound peptides into gramicidin S in the absence of enzyme II*

Of the two enzymes involved in gramicidin S synthesis, enzyme I which is the larger and contains one mole of 4′-phosphopantetheine [6, 7] activates proline, valine, ornithine and leucine, and enzyme II which is the smaller, activates and racemizes phenylalanine. In order to decide whether enzyme I when charged with intermediate peptides, can convert these into gramicidin S in the absence of enzyme II, the following experiment was carried out.

Gramicidin S synthetase was incubated with ^{14}C-phenylalanine, unlabelled proline, valine, ornithine (leucine absent) and ATP, and the mixture was fractionated on Sephadex G-200 at pH 6.0 to separate enzyme I from enzyme II. A typical elution pattern is seen in fig. 1. About 80% of the total radioactivity in enzyme I (first peak) is bound to the protein. Unexpectedly, about 20% of the total radioactivity was soluble in TCA (see fig. 1). The most likely explanation for this is that some splitting off of covalently bound intermediate peptides occurs during the fractionation procedure. The two most radioactive fractions of enzyme I (fractions 19 and 20, see fig. 1) were incubated in the presence of unlabelled phenylalanine, proline, valine, ornithine, leucine and ATP. Table 1 shows the result of two separate experiments. It is seen that there is substantial incorporation of protein bound radioactivity into gramicidin S without addition of enzyme II. The fractions used (19 and 20, see fig. 1) contained only trace amount of enzyme II. This was shown by measuring the ATP–^{32}P-pyrophosphate exchange reaction with L-Phe as substrate, and comparing it to that obtained with L-Leu. The result agreed well with our purest enzyme I preparation described previously [6] where only trace amount of enzyme II was detectable after disc electrophoresis.

Since 16–24% (table 1) of the enzyme bound radioactivity is converted into gramicidin S and since only about 4% of the protein bound radioactivity was thioester bound phenylalanine, the experiments show that enzyme I charged with intermediate peptides can convert part of these into gramicidin S in the absence of enzyme II.

Experiments were also carried out where addition of enzyme II containing no labelled phenylalanine was added to enzyme I charged with peptide intermediates. It is seen from table 1 and fig. 2 that this increased somewhat the degree of incorporation of radioactivity into gramicidin S.

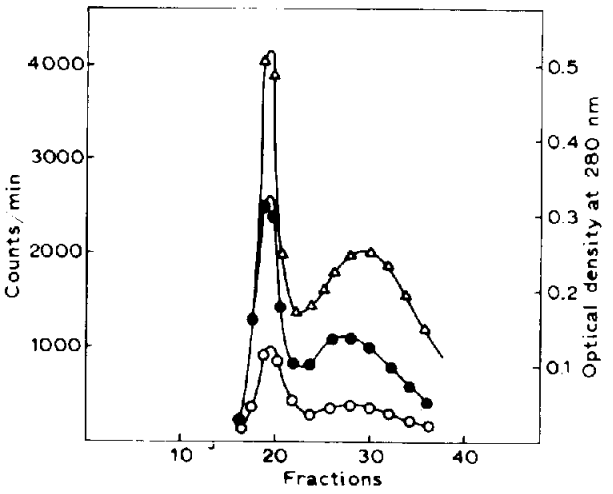

Fig. 1. Fractionation of gramicidin S synthetase on a Sephadex G-200 column (2.5 X 38 cm) after loading the enzyme with intermediate peptides in the absence of L-leucine. Gramicidin S synthetase (25 mg) was incubated in a total volume of 3 ml for 3 min at 37° for containing 0.02 mM ^{14}C-L-phenylalanine (160 Ci/mole), 0.05 mM unlabelled L-proline, L-valine, L-ornithine, 0.1 M triethanolamine hydrochloride buffer (pH adjusted to 7.5), 10 mM magnesium chloride, 5 mM KCl, 2.5 mM ATP and 6 mM β-mercaptoethanol. The mixture was cooled to 0°, put on the column and eluted at 4° with a 25 mM potassium citrate buffer (pH adjusted to 6.0 at room temperature), containing 20% glycerol, 0.25 mM EDTA and 1 mM DTT. Fractions of 2.5 ml were collected and the optical density at 280 nm (△——△) was measured. The radioactivity in the protein fractions (●——●) together with the radioactivity soluble in 5% TCA (○——○) were determined.

The rate of incorporation of the enzyme I bound peptides into gramicidin S under these conditions was also measured. It is seen from fig. 2 that the incorporation is complete after 1 min and that there is a decrease in the protein bound radioactivity. In addition, there is also an increase in the amount of TCA soluble radioactivity. Presumably, part of the enzyme bound peptides is liberated without being converted into gramicidin S.

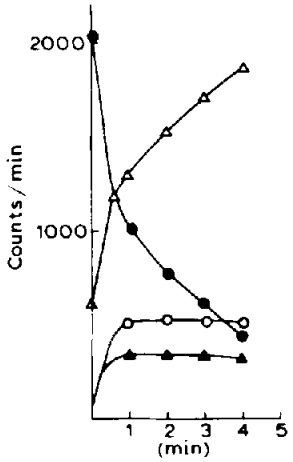

Fig. 2. Incorporation of radioactivity into gramicidin S after reincubation for various periods of time of the pooled enzyme I fraction obtained after fractionation on Sephadex G-200 as described in fig. 1. The reincubation mixture was identical to that described under table 1 and was carried out in the absence and presence of enzyme II. ▲—▲ and ○—○ represent incorporation into gramicidin S in the absence and presence of enzyme II. In addition, the protein bound radioactivity (●—●) and the TCA soluble radioactivity (△—△) in the presence of enzyme II are indicated.

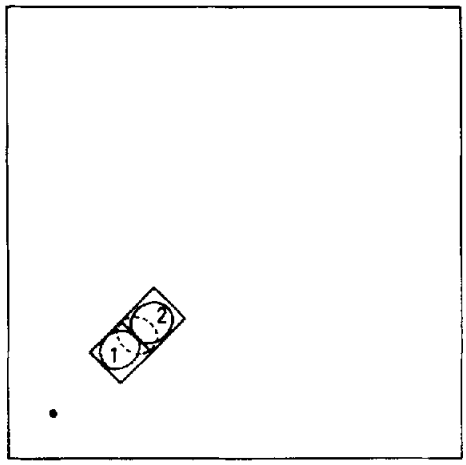

Fig. 3. The position of gramicidin S (2), gramicidin S where proline is replaced by azetidine-2-carboxylic acid (1) and the hybrid decapeptide (dotted circle) and a two dimensional thin-layer chromatogram with ethyl acetate–pyridine–acetic acid–water in both dimensions.

3.2. *Cyclisation mechanism*

In order to decide whether two pentapeptides attached to the same enzyme I molecule or pentapeptides attached to separate enzyme I molecules cyclize, we took advantage of the fact that gramicidin S synthetase can use azetidine-2-carboxylic acid instead of proline. If the reaction is intramolecular, mixing two aliquotes of enzyme I charged with peptides containing respectively azetidine-2-carboxylic acid and proline should yield no hybrid decapeptide (containing one molecule of proline and one of azetidine-2-carboxylic acid). If the reaction is intermolecular, 50% of the decapeptide formed would be expected to be hybrid. Previous experiments had shown that the hybrid in thin-layer chromatography occupies a position which is intermediate between gramicidin S and the analogous decapeptide containing azetidine-2-carboxylic acid (see fig. 3). Two separate incubation mixtures of gramicidin S synthetase containing ^{3}H-proline and ^{14}C-azetidine-2-carboxylic acid respectively and in addition phenylalanine, valine, ornithine (no leucine) were fractionated on Sephadex G-200 columns and the two enzyme I fractions were combined and incubated after addition of phenylalanine, proline, valine, ornithine, leucine and ATP (see table 2). The labelled decapeptides formed were separated by thin-layer chromatography and the plate developed by radioautography. The areas corresponding to the position of gramicidin S and the analogous decapeptide containing azetidine-2-carboxylic acid were removed and counted. It appears from table 2 that the area corresponding to the position of gramicidin S contained 91% of the total ^{3}H label and was only contaminated with 5% of the total ^{14}C label. The spot corresponding to the position of the decapeptide with azetidine-2-carboxylic acid contained 95% of the total ^{14}C label and only 9% of the ^{3}H label. If the cyclisation reaction took place between separate enzyme molecules, then as mentioned, 50% of the decapeptide formed would be expected to be hybrid. The small amounts of the other isotope (5 and 9%) in the two areas mentioned above are not more than could be accounted for by a small overlap of the gramicidin S spot and the spot containing azetidine-2-carboxylic acid (see fig. 3). Hence the results strongly suggest that two activated peptides on the same molecule undergo cyclisation.

Table 1
Reincubation of enzyme I fraction obtained from the Sephadex G-200 experiment.

Experiment	Enzyme II	Radioactivity incorporated into gramicidin S (cpm)	Radioactivity in gramicidin S as percentage of	
			total radioactivity	protein bound radioactivity
1	Absent	1190	10.6	16.0
	Added	1680	15.0	22.7
2	Absent	3050	15.8	24.4
	Added	4060	20.9	32.5

The two most radioactive fractions (first peak see fig. 1) were pooled and while kept at 0°, added 0.05 mM of L-phenylalanine, L-proline, L-valine, L-ornithine and L-leucine, 0.1 M triethanolamine hydrochloric acid (final pH 7.5), 50 mM magnesium chloride (under these conditions citrate does not interfere with the gramicidin S formation) 5 mM KCl, 6 mM β-mercaptoethanol and 1 mg albumin per ml. An aliquote which was immediately precipitated with TCA was used as a blank. To the remaining mixture was added solid ATP at a final concentration of 2.5 mM and the mixture was incubated at 37° for 1 min and the incorporation into gramicidin S was determined. Enzyme II originated from a different enzyme preparation and had been separated from enzyme I by Sephadex G-200 chromatography.

Table 2
Examination of cyclic decapeptides produced when mixing two enzyme I preparations charged with peptides containing respectively ^{3}H-L-proline and ^{14}C-L-azetidine-2-carboxylic acid.

	^{14}C radioactivity (cpm)	^{3}H radioactivity (cpm)
Square corresponding to position of gramicidin S where proline was completely replaced by azetidine-2-carboxylic acid	8886	933
Square corresponding to position of gramicidin S	486	9732
Sum	9372	10665

Two equal parts of gramicidin S synthetase (20 mg) were incubated separately in a total volume of 3 ml for 3 min at 37° with respectively 0.05 mM ^{3}H-L-proline (30 Ci/mole) and 0.1 mM ^{14}C-D,L-azetidine-2-carboxylic acid (10 Ci/mole) and the following: 0.05 mM of L-phenylalanine, L-valine, L-ornithine, 0.1 M triethanolamine buffer (pH 7.5), 10 mM magnesium chloride, 5 mM KCl, 2.5 mM ATP and 6 mM β-mercaptoethanol. The mixtures were quickly cooled to 0° and fractionated simultaneously on separate Sephadex G-200 columns as described in fig. 1. The pooled enzyme I fractions from both columns were mixed, kept at 0°, and 0.05 mM of L-phenylalanine, L-proline, L-valine, L-ornithine, L-leucine, 0.1 M triethanolamine hydrochloric acid (final pH 7.5), 50 mM magnesium chloride and 2.5 mM solid ATP were added to be mixture. The mixture was then incubated at 37° for 1 min. The decapeptides formed were fractionated by two dimensional thin-layer chromatohraphy (fig. 3) and the plate developed by radioautography. The two squares indicated (see fig. 3) were removed and counted.

4. Discussion

The experiment described in table I provides very good evidence that when enzyme I is loaded with the intermediate peptides Phe–Pro, Phe–Pro–Val and Phe–Pro–Val–Orn [2, 3], then enzyme II is not required for the formation of gramicidin S. However, the total amount of radioactivity incorporated into gramicidin S was always larger if unlabelled enzyme II was added. An explanation for this may be that the presence of enzyme II will initiate the growth of new unlabelled peptide chains. Since the present experiments show that the cyclisation reaction occurs between two pentapeptides on the same enzyme molecule, initiation of new chains would be expected to lead to a more efficient conversion of labelled peptides into gramicidin S. The reason why not all protein bound peptides can be converted into gramicidin S may be that during the fractionation procedure part of the protein loses its ability to convert the protein bound intermediates (fig. 2) into gramicidin S, and results in the splitting off from the enzyme of intermediate peptides (TCA soluble).

The data presented support strongly the view that the cyclisation of two pentapeptides takes place on the same enzyme molecule. However, it has not escaped our attention that if two enzyme molecules aggregated to a dimer when charged with intermediates, and remained aggregated after mixing of the two incubation mixtures (see table 2) our conclusion would not be correct. No such dimer had, however, been detected in the analytical ultracentrifuge.

As pointed out previously [6] the enzyme contains only one 4′-phosphopantetheine arm and it seems possible that the growing peptide chain is linked through a thioester linkage to the 4′-phospho-pantetheine arm. Since the cyclisation reaction is an intramolecular reaction, the enzyme would then have to transfer the pentapeptide from the arm to another thiol group where it "sits and waits" while the 4′-phosphopantetheine arm moves back and then participates in the formation of another pentapeptide. This second pentapeptide, while linked to the arm, could be brought in such close contact with the waiting pentapeptide that head to tail cyclisation occurs.

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