REPLACEMENT OF PHENYLALANINE IN GRAMICIDIN S BY OTHER AMINO ACIDS

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1. Introduction

Gramicidin S is a cyclic decapeptide produced by *Bacillus brevis* with a repeating sequence of 5 amino acids joined head to tail:

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

This decapeptide is synthesized by gramicidin S synthetase which consists of two enzymes, the light and the heavy [1]. The light enzyme which is also a racemase, activates and thioesterbinds phenylalanine. The growth of the peptide chain is initiated by transfer of the thioester-bound D-phenylalanyl group to the heavy enzyme. This enzyme activates and thioesterbinds L-proline, L-valine, L-ornithine and L-leucine and catalyzes the formation of the peptide bonds in gramicidin S.

In the past, substitution of phenylalanine in gramicidin S by other amino acids using gramicidin S synthetase has been claimed to take place. For instance it has been reported that *p*-fluorophenylalanine and β-thienylalanine will substitute [2]. However, in neither case have the cyclic decapeptides been identified chromatographically and separated from gramicidin S. Furthermore, there is conflicting evidence on whether or not tyrosine can replace phenylalanine [2,3]. It has also been claimed that tryptophan could not replace this amino acid [3]. We have re-examined these reports and present evidence that *p*-fluoro-, *p*-chloro-, *p*-bromophenylalanine, β-thienylalanine, tyrosine and tryptophan can replace phenylalanine. Evidence for the formation of a hybrid decapeptide containing one residue of tyrosine and one of phenylalanine is also presented.

In addition, cyclohexylalanine, phenylglycine and α-methyl phenylalanine have been examined for their ability to substitute for phenylalanine. None of these could substitute in the synthesis. However, it was found that in the presence of cyclohexylalanine and proline only, synthesis is initiated since the dipeptide cyclohexylalanylproline is produced. If valine and leucine were present, the formation of the dipeptide was inhibited. This probably explains why no synthesis of the cyclic decapeptide takes place in a complete incubation mixture.

2. Methods

2.1. *Growth of organism*

B.brevis ATCC 9999 was cultivated as in [4].

2.2. *Preparation of the light and heavy enzyme of gramicidin S synthetase*

The method used was that in [4].

2.3. *ATP–$^{32}PP_i$ exchange reactions*

The reaction was carried out according to [5] in 0.1 ml total vol. The radioactivity in ATP was determined by the methods in [6].

2.4. *Thioesterbinding of amino acids to light enzyme*

This was carried out as in [7].

2.5. *Synthesis of cyclic decapeptides*

Synthesis was determined by the Millipore filter assay [8]. Each assay contained in 0.2 ml total vol.:

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20 mM sodium phosphate (pH 7.2); 5 mM ATP; 25 mM $MgCl_2$; 5 mM dithiothreitol; 6.25 μM EDTA; 1 mM of the amino acid to replace phenylalanine; 0.05 mM L-[^{14}C]proline (20 Ci/mol); L-valine; L-ornithine; L-leucine; the mixture was incubated at 37°C for 10 min. The incubation mixture was precipitated with 0.1 ml 20% trichloroacetic acid (TCA) containing 0.1 M L-proline and washed repeatedly with 5% TCA and 2% Na_2SO_4. The precipitate was extracted with ethanol–0.2 M HCl (9:1, v/v) and the extract concentrated. An aliquot was used for counting and the remainder subjected to thin-layer chromatography together with authentic gramicidin S on DC-Alufolien Kieselgel 60 (Merck) in the solvent: ethylacetate–pyridine–acetic acid–water (60:20:6:11, by vol.). The plate was subjected to radioautography then sprayed with ninhydrine.

2.6. *Large scale synthesis and amino acid analysis of cyclic decapeptide containing tyrosine*

The incubation mixture contained in 6 ml total vol., 3 ml of the 40% $(NH_4)_2SO_4$ fraction of gramicidin S synthetase [5]. Otherwise the incubation mixture was as above except that amino acid was 1 mM and L-[^{14}C]proline was spec. act. 0.1 mCi/mmol. The mixture was incubated for 60 min at 37°C and the cyclic decapeptide extracted as above. The chromatographically purified peptide was hydrolyzed in a sealed tube in 1 ml 6.0 N HCl containing 0.05% mercaptoacetic acid and amino acid analysis carried out using a Bio Cal BC-200 amino acid analyzer. The yield of cyclic decapeptide by the in vitro synthesis was ~150 nmol.

3. Materials

3.1. *Amino acids*

L-Phenylalanine, L-tyrosine, D,L-tryptophan, *p*-bromo-D,L-phenylalanine and β-thienyl-D,L-alanine were from Sigma. *p*-Fluoro-D,L-phenylalanine and *p*-chloro-D,L-phenylalanine were from Koch-Light. D,L-phenylglycine and L-tryptophan were purchased from Mann Res. Labs. α-Methyl-D,L-phenylalanine and D,L-tyrosine were obtained from Nutritional Biochem. Corp. Cyclohexyl-D,L-alanine was synthesized from D,L-phenylalanine by the method in [9].

3.2. *Labelled amino acids*

L-[^{14}C]Proline (270 mCi/mmol) and L-[^{14}C]-phenylalanine (500 mCi/mmol) were purchased from Amersham. L-[^{14}C]Cyclohexylalanine was synthesized from L-[^{14}C]phenylalanine by the method above and purified by the thin-layer system used for cyclic decapeptides.

4. Results and discussion

All the amino acids chosen for these studies were initially examined for the ATP–$^{31}PP_i$ exchange reaction by the light enzyme. The results are given in table 1. It was found that the reaction occurred to a significant degree in the presence of D,L-tyrosine, D,L-tryptophan, cyclohexyl-D,L-alanine, β-thienyl-D,L-alanine and *p*-bromo-D,L-phenylalanine, respectively. However, no exchange reaction took place in the presence of D,L-phenylglycine and α-methyl-D,L-phenylalanine. The amino acids which became activated were then studied for their ability to replace phenylalanine in the biosynthesis of the cyclic decapeptide. All except cyclohexylalanine were incorporated (table 1) to varying degrees. The [^{14}C]-proline-labelled reaction products were examined by thin-layer chromatography and radioautography. A schematic summary of the results is presented in fig.1, positions 1–7. With one exception (tryptophan), the radioactive products formed have mobilities relative to normal gramicidin S corresponding to the relative mobilities of the substituting amino acids to that of phenylalanine. If the substituting amino acids had a higher mobility than phenylalanine, the product would have a higher mobility and the other way round. In the case of tryptophan which has the same mobility as phenylalanine in the chromatographic system used, the reaction product has a slightly lower mobility than that of normal gramicidin S.

The labelled peptide obtained with D,L-tyrosine was prepared in sufficient quantities and subjected to amino acid analysis. It contained, as expected, the amino acids in the following molar proportions: tyrosine/proline/valine/ornithine/leucine = 1.00/1.00/1.03/1.07/1.16.

When L-tyrosine was used instead of D,L-tyrosine, 3 labelled spots were detected (fig.1, position 8). One corresponds to gramicidin S, one to the position of

Table 1
ATP–$^{32}PP_i$ exchange reaction and in vitro synthesis of cyclic decapeptides in the presence of amino acid analogues of phenylalanine

Amino acid	ATP–$^{32}PP_i$ exchange reaction (%)	Synthesis of cyclic decapeptide (%)
D,L-phenylalanine (control)	100	100
D,L-tyrosine	14	60
p-fluoro-D,L-phenylalanine	41	26
p-chloro-D,L-phenylalanine	25	13
p-bromo-D,L-phenylalanine	8	7
D,L-tryptophan	12	17
β-thienyl-D,L-alanine	54	58
cyclohexyl-D,L-alanine	38	0
D,L-phenylglycine	0	–
α-methyl-D,L-phenylalanine	0	–

In decapeptide synthesis the concentration of the amino acids substituting for phenylalanine was 1 mM. Because of the low solubility of *p*-bromo-D,L-phenylalanine, the solid was added to the incubation mixture and hence its concentration is not known. For the exchange reaction the concentration of the amino acids was 2 mM except for D,L-tyrosine. In this case it was 1 mM as a result of low solubility. For further details see section 2

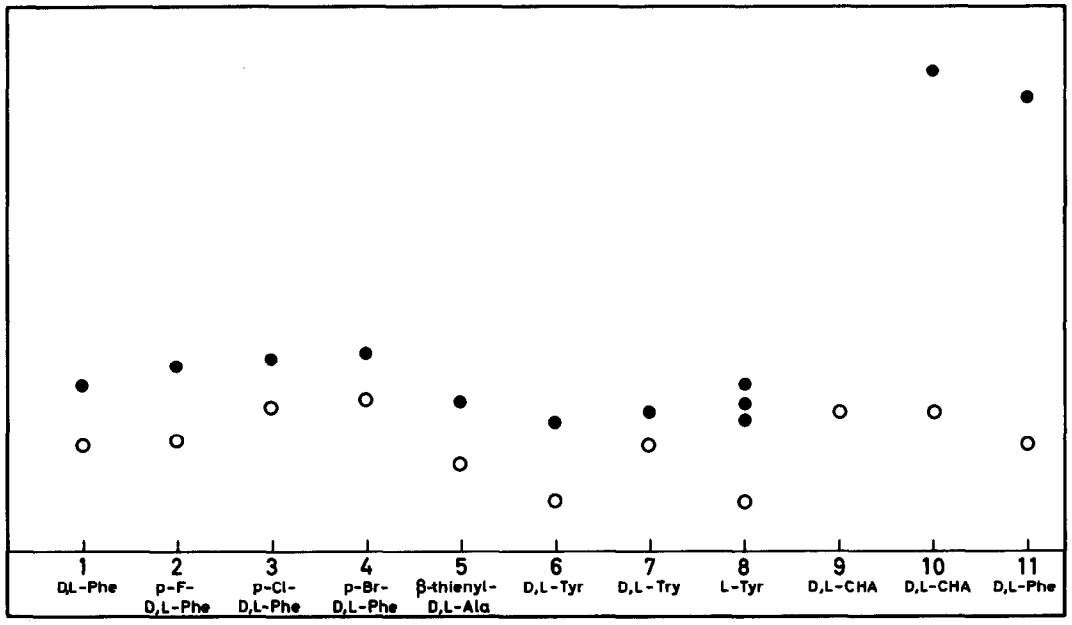

Fig.1. Summary of results from thin-layer chromatograms of labelled peptides formed when replacing phenylalanine with other amino acids. Filled circles indicate position of labelled peptides (labelled with L-[^{14}C]proline) and open circles amino acids (phenylalanine or amino acids replacing phenylalanine). Position 1 is the control giving the position of phenylalanine and gramicidin S. Positions 2–9 give results from incubation mixtures where phenylalanine has been replaced with the amino acids indicated on the chromatogram. Positions 10 and 11 indicate peptides formed when only L-[^{14}C]proline and D,L-cyclohexylalanine or D,L-phenylalanine, respectively, were present in the incubation mixture. CHA, cyclohexylalanine.

the cyclic decapeptide containing 2 tyrosine residues, and the third spot occupied an intermediate position. This was taken as an indication of the possible contamination of the L-tyrosine with phenylalanine and suggested that the intermediate spot could be a hybrid containing 1 tyrosine and 1 phenylalanine residue. Amino acid analysis of the L-tyrosine sample revealed that it contained ~1% phenylalanine.

Cyclohexylalanine, as shown in table 1 and fig.1, position 9, did not support synthesis of any cyclic decapeptide. It has, however, been found that L-[^{14}C]-cyclohexylalanine becomes thioester-bound to the light enzyme (results not shown). When incubating gramicidin S synthetase with D,L-cyclohexylalanine and L-[^{14}C]proline or L-[^{14}C]cyclohexylalanine (20 mCi/mmol) and L-proline, a labelled peptide was liberated into the ethanol–HCl extract (position 10, fig.1). By comparing the mobilities, this peptide corresponds to the D-phenylalanyl-proline diketo-piperazine (position 11, fig.1) which is known to be liberated by ethanol–HCl. The experiments show that cyclohexylalanine may be transferred from the light to the heavy enzyme under these conditions.

The reason why no peptide is produced with cyclohexylalanine in an otherwise complete incubation mixture seems to be inhibition of cyclohexylalanine utilization by the hydrophobic amino acids valine and leucine. This was shown by the fact that when D,L-cyclohexylalanine and L-[^{14}C]proline were incubated in the presence of 0.05 mM L-leucine and 0.05 mM L-valine, no labelled dipeptide was formed.

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