BACITRACIN BIOSYNTHESIS BY THREE COMPLEMENTARY FRACTIONS FROM *BACILLUS LICHENIFORMIS*

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1. Introduction

The non-ribosomal synthesis of the dodecapeptide bacitracin in a cell free system has been reported by Ishihara et al., Simlot et al., and Frøyshov and Laland [1–3]. These experiments [3,4] indicate that its mechanism of synthesis is in principle similar to the 'thiotemplate mechanism' for the synthesis of gramicidin S and tyrocidine [5]. The amino acids are activated through the formation of aminoacyladenylates and then transferred to thioester sites before polymerization on the enzyme. Ishihara and Shimura have demonstrated the presence of two enzymes [4], which activate the amino acids in the linear and the cyclic part of bacitracin respectively. Frøyshov and Laland have demonstrated the presence of two enzymes [3]. One of them activates all the amino acids in bacitracin except lysine and ornithine. These two amino acids from the cyclic part of the molecule are activated by the other enzyme. Both enzymes are necessary for synthesis of bacitracin. In the present work, bacitracin synthetase from *Bacillus licheniformis* ATCC 10716 was resolved into three complementary fractions by affinity chromatography, on a column containing L-leucine bound through its carboxyl group to the amino group of amino-alkyl-Sepharose. The first fraction activates the amino acids in the linear part of the molecule. The second activates lysine and ornithine, and the third activates isoleucine and the remaining amino acids in bacitracin.

2. Methods and materials

2.1. *Bacitracin synthetase*

The 43–49% satd. $(NH_4)_2SO_4$ fractions prepared as described by Frøyshov and Laland [3] from *Bacillus licheniformis* ATCC 10716 were used in the experiments.

2.2. *Preparation of column*

The affinity chromatography column (3 × 1.6 cm) was prepared from 2 g Sepharose 4-B (Pharmacia Fine Chemicals) and 3,3′-diaminodiproplylamine, with L-leucine as ligand as described by Pass et al. [6]. The elution buffer called buffer B contained 0.05 M potassium phosphate, pH 7.5, 0.2 mM $MgCl_2$ and 0.1 mM dithiothreitol.

2.3. *Estimation of ATP–[^{32}P]PP; exchange reaction*

The incubations were carried out at 37°C for 30 min and the exchange reaction was determined as described previously [3] with one exception, the concentrations of L-Asp and L-Glu were 20 μmole/ml incubation mixture [4].

2.4. *Estimation of bacitracin synthesis*

The incubations were carried out at 37°C for 15 min and the amount of bacitracin synthesis was determined [3] using thin-layer chromatography and radioautography.

2.5. *Labelled substances and measurement of radioactivity*

L-[U-^{14}C] isoleucine and [^{32}P]$Na_4P_2O_7$ were obtained from New England Nuclear Corp., Boston, and from The Radiochemical Centre, Amersham. For counting a Packard Tri-Carb Liquid Scintillation counter or a Frieseke and Hoepfner gas-flow counter was used.

3. Results

In the experiments the Sepharose column used contained as ligand L-leucine, bound through its carboxyl group to the 3,3′-diaminodiprophylamine spacer. Leucine is one of the amino acids in the linear part of bacitracin. When bacitracin synthetase (the 43–49% satd. $(NH_4)_2SO_4$ fraction) was applied to the column, it was retained and could be eluted by the addition of 0.1–0.4 M KCl to the buffer. All fractions were monitored for the ATP–[^{32}P]PP_i exchange reaction in the presence of the amino acids occurring in bacitracin A (see fig. 1.). It is seen that all the amino acids (under the condition used) are activated in one of the three peaks designated A, B and C. Peak A activates L-Ile, L-Cys, L-Leu and L-Glu. Peak B activates L-Lys and L-Orn. Peak C activates L-Phe, L-His, L-Asp and L-Asn. The shoulder found in the L-Ile-dependent exchange activity profile (fraction 16 and 17 fig. 1) indicates that Peak C also activates L-Ile. The yield of the three enzymes after fractionation (as determined by the exchange reactions) was 90–100%. All three fractions were required for maximal bacitracin synthesis (see table 1), and a combination of any two fractions was less than 25% as active as the three fractions combined. The L-leucine dependent exchange reaction catalyzed by fractions 7–11 (see fig. 1) is probably due to the presence of leucyl-tRNA synthetase since

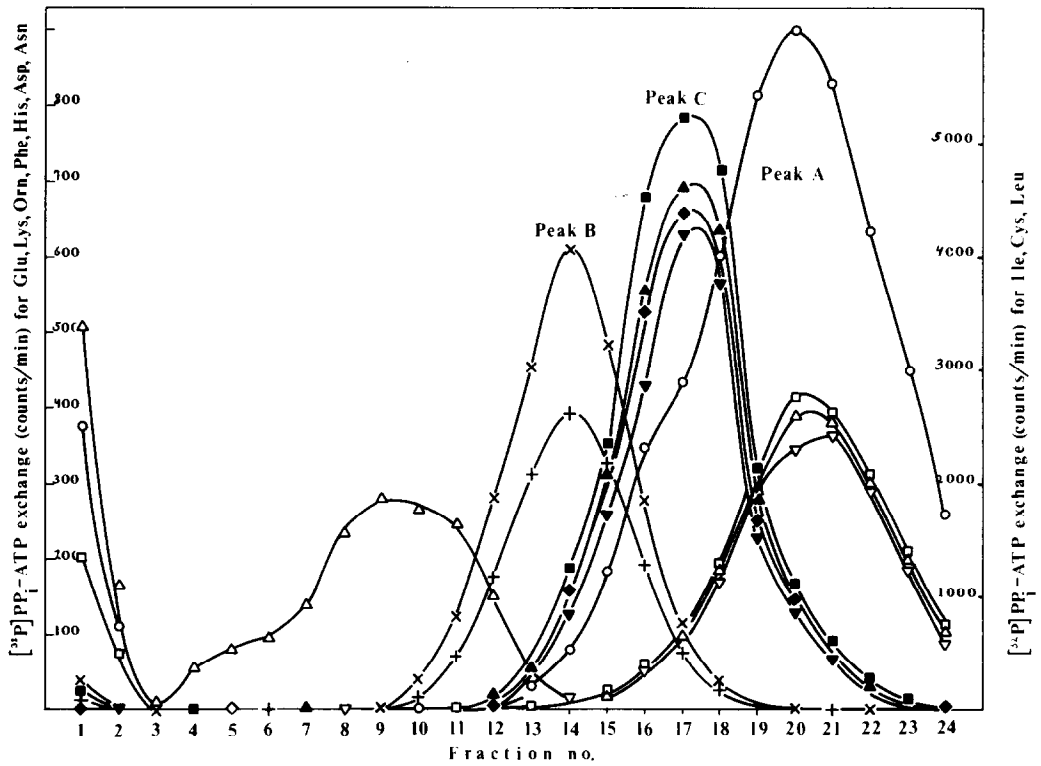

Fig. 1. Fractionation of bacitracin synthetase on a Sepharose column using L-leucine as a ligand. 0.25 ml (1.5 mg protein) of the 43–49% $(NH_4)_2SO_4$ fraction obtained from 2.5 g wet cells (0.5 l culture) was put on a column (3 × 1.6 cm) equilibrated with buffer B at 4°C. The column was then eluted with buffer B (60 ml/hr). The volume of the fractions was 6.5 ml. After fraction no. 2, 0.1 M KCl was added to the buffer. After fraction no. 8, an 0.1–0.4 M linear KCl gradient in buffer B was used. [^{32}P]PP_i–ATP exchange activity dependent on the L-amino acids isoleucine (○), cysteine (□), leucine (△), glutamic acid (▽), lysine (+) ornithine (×), phenylalanine (▼), histidine (■), aspartic acid (▲), and asparagine (◆), was measured in 0.2 ml aliquots of each fraction. Total amount of radioactivity in each incubation mixture was 120 000 cpm. The radioactivity (40–50 cpm), measured in control tubes (without any amino acid), was subtracted. The exchange activities for isoleucine, cysteine and leucine given in the figure have been reduced by a factor of 7 for the purpose of convenience.

Table 1
Requirement for three enzyme fractions in bacitracin biosynthesis

Added fraction	Bacitracin synthesis (cpm)
A + B + C	2160
A + B	450
A + C	315
B + C	535
A	150
B	210
C	440

To the contents of tubes 12–14, 16–17 and 18–20 (fig. 1) were added 2 mg bovine serum albumin each. Solid $(NH_4)_2SO_4$ was added to 80% saturation and the precipitate dissolved in 0.2 ml buffer B. The fractions were designated Peak B, C, and A respectively. Bacitracin formation was measured as described with 20 μl of Peak A, Peak B and Peak C. The bacitracin synthesizing activity was measured after 15 min incubation using L-[^{14}C] isoleucine (100 000 cpm). Zero time incubation for all the different incubation mixtures was 165–175 cpm.

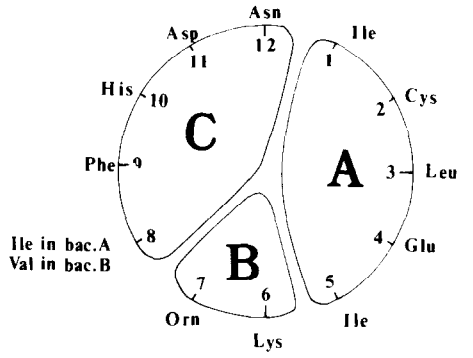

Fig. 2. Schematic representation of bacitracin synthetase. A, B and C are the three peaks coming out of the column which are necessary for bacitracin biosynthesis. The amino acids in bacitracin A (and B) are numbered 1–12 from the N-terminal end of the molecule. For further details see text.

leucine was the ligand on the column. No stimulation of bacitracin synthesis was achieved by adding concentrated protein from fractions 7–11 to the combined Peaks A, B, and C (results not shown).

4. Discussion

The present results provide good evidence that bacitracin synthetase consists of at least three different enzymes. The enzyme present in Peak A (fig. 1.), activates L-isoleucine, L-cysteine, L-leucine and L-glutamic acid. The enzyme present in Peak B activates L-lysine and L-ornithine. The enzyme present in Peak C activates L-isoleucine, L-phenylalanine, L-histidine, L-aspartic acid and L-asparagine. Peak C also activates L-valine, which is found in position no. 8 from the N-terminus in bacitracin B as indicated on fig. 2. (Results not shown.) L-Isoleucine in positions no. 1 and 5 is probably activated by Peak A since Peak B (the next enzyme taking part in the polymerisation?) only activates L-lysine and L-ornithine. No enzyme with single 'isoleucine activity' was found.

It seems likely that enzyme A (Peak A) was retained by the column because it activates leucine and hence shows affinity for the leucine ligand. Enzyme C is probably retained because it shows affinity for enzyme A. Enzyme B is probably retained because it shows affinity for either enzyme A or C. The presence of ATP was not required for binding of the enzymes to the column. This is in contrast to what is found for gramicidin S synthetase where only a little binding of the heavy enzyme took place to the 'leucine column' in the absence of ATP [6]. By using different substrate amino acids as ligands on columns, it should be possible to study the interaction and the location of the areas on the enzymes in bacitracin synthetase which exhibit affinity for each other.

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