ON THE PRESENCE OF PANTOTHENIC ACID IN THE THREE COMPLEMENTARY ENZYMES OF BACITRACIN SYNTHETASE

Ingrid ROLAND, Øystein FRØYSHOV,
Department of Research and Development, Apothekernes Laboratorium for Specialpræparater, Oslo 2,

and

Søren G. LALAND
Department of Biochemistry, University of Oslo, Blindern, Oslo 3, Norway

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1. Introduction

Recent studies on the biosynthesis of bacitracin have shown that this peptide is synthesized by a non-ribosomal peptide synthesis [1–5], similar to the thiotemplate mechanism for gramicidin S and tyrocidine [6]. Since phosphopantetheine is involved in the synthesis of the two latter peptides, partially purified bacitracin synthetase prepared in this laboratory was examined for its presence [4]. It was found that pantothenic acid was liberated from the enzyme preparation upon alkaline and phosphatase* treatment, and that the amount liberated compared quite well with the amount of enzyme bound substrate amino acids. In order to obtain further information about the presence of pantothenic acid in bacitracin synthetase, a pantothenic acid-requiring mutant has been prepared and the incorporation of labelled pantothenic acid into bacitracin synthetase separated into its three complementary components [7] has been studied. Furthermore, the amount of pantothenic acid liberated from each of the three components was estimated and compared with the amount of enzyme-bound substrate amino acids.

*Alkaline phosphatase: EC 3.1.3.1.

2. Materials and methods

2.1. *Preparation of a pantothenic acid-requiring mutant*

A pantothenic acid-requiring mutant of *Bacillus licheniformis* ATCC 10716 was prepared after u.v. radiation of spores. The mutant yielded the same amount of bacitracin as the parent strain.

2.2. *Growth of the mutant*

Spores of the mutant was added to 50 ml of medium which contained: 2% L-glutamic acid, 0.02% L-valine, 0.2% NaH_2PO_4, 0.1% citric acid, 0.05% KCl, 0.05% Na_2SO_4, 0.02% $MgCl_2 \cdot 6H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.01% $Fe_2SO_4 \cdot 7H_2O$, 0.01% $MnSO_4 \cdot H_2O$ and 0.5 mg of the calcium salt of pantothenic acid. The pH of the medium was adjusted to 7.0 with NaOH. The mixture was incubated in a New Brunswick rotary shaker at 37°C for 16 h. 0.5 ml of this culture was used to inoculate 250 ml of the medium containing 1.75 mg of [^{3}H]pantothenic acid (approx. 0.4 mCi) in each flask. The cells were grown at 37°C in the shaker and were harvested in the late logarithmic phase (absorbance at 650 nm = 2.3 in Spectronic 20) and washed once with 0.04 M potassium phosphate buffer pH 7.2 containing 2 mM $MgSO_4$.

2.3. *Preparation of partially purified cell-free extract*

The 43–49% $(NH_4)_2SO_4$ fraction containing bacitracin synthetase was prepared as described previously [4].

2.4. *Fractionation of bacitracin synthetase into its three complementary enzymes A, B and C*

The 43–49% $(NH_4)_2SO_4$ fraction was fractionated on a Sepharose column containing L-leucine bound through its carboxyl group to the 3,3′-diamino-dipropylamine spacer as described by Frφyshov [7].

2.5. *Estimation of the ATP–[$^{32}PP_i$]exchange reaction*

The incubation was carried out as described previously [4] with the exception that an incubation period of 30 min was used.

2.6. *Release of pantothenic acid from the enzyme and its microbiological assay*

This was carried out as described by Gilhuus-Moe et al. [8] using *Lactobacillus plantarum.*

2.7. *Preparation of bacitracin synthetase for the purpose of microbiological estimation of pantothenic acid*

B. licheniformis ATCC 10716 was grown and the cell-free extract prepared as described previously [4].

2.8. *Preparation of [^{3}H]pantothenic acid*

Tritiated pantothenic acid was synthesized from labelled β-alanine as described by Wieland et al. [9]. The labelled pantothenic acid was purified by paper chromatography in propan-2-ol–formic acid–water (75:10:15 v/v/v).

2.9. *Estimation of enzyme bound amino acids (thioester-bound)*

This was carried out as described previously [4].

2.10. *Labelled substances and measurement of radioactivity*

β-[3-^{3}H(N)]alanine (spec. act. 37.5 Ci/mmole) and ^{14}C-labelled amino acids were purchased from New England Nuclear Corp., Boston, and [^{32}P]$Na_4P_2O_7$ 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 and discussion

Bacitracin synthetase labelled with [^{3}H]pantothenic acid was obtained from the pantothenic acid-requiring mutant of *B. licheniformis* (see Materials and methods) and separated into the three enzymes A, B and C as shown in fig.1. It is seen that there is a peak of radio-

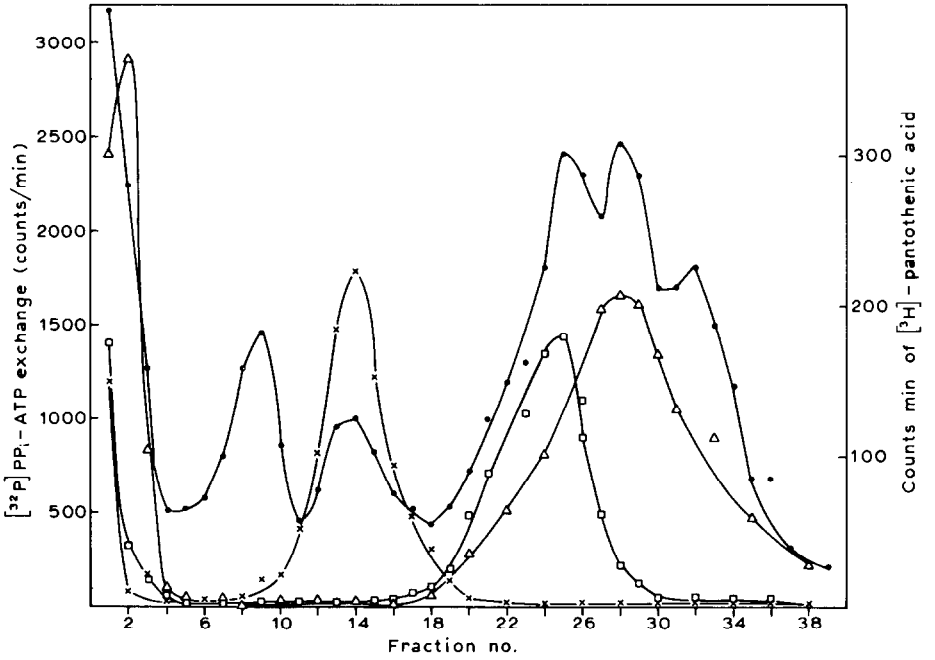

Fig.1.

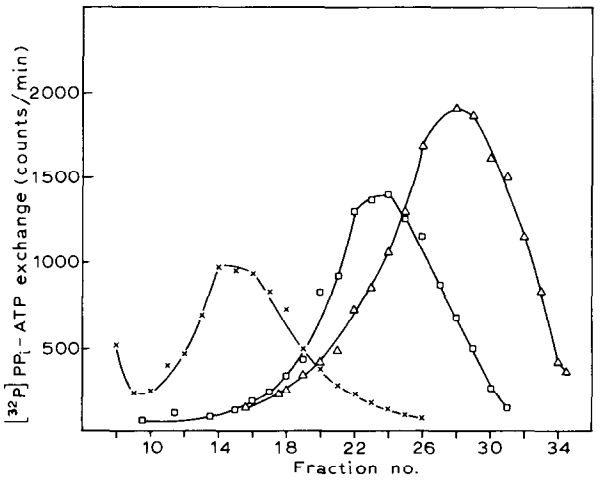

Fig.2. Separation of enzymes A, B and C on the Sepharose column (see Materials and methods) for the microbiological estimation of pantothenic acid. A column (5.5 × 2.6 cm) equilibrated with buffer B (see fig.1) was used. Bacitracin synthetase (43–49% $(NH_4)_2SO_4$ fraction) was put on the column and eluted with buffer B (30 ml/h). After fraction No. 1. 0.1 M KCl was added to the buffer followed by a 0.1–0.4 M KCl gradient in buffer B at fraction No. 6. The volume of each fraction was 15 ml. The positions of the three enzymes were located using the amino acid $^{32}PP_i$–ATP exchange activity as described in fig.1. For the microbiological estimation of pantothenic acid fractions 10–18, 18–24 and 26–34 respectively were combined and used.

activity corresponding exactly to the position of respectively enzyme A, B and C. Thus, all three components seem to contain pantothenic acid. A certain amount of bacitracin synthetase is not retained by the column and this explains partly why not all radioactivity was retained. In order to demonstrate microbiologically the presence of pantothenic acid in enzymes A, B and C larger quantities of these were prepared using the parent strain ATCC 10716. Enzymes A, B and C were separated on a Sepharose column in several runs which gave nearly identical results. On fig.2, which shows a typical run, is indicated which parts of respectively A, B and C were used for the microbiological determination. An aliquot of each was used for estimating the

Table 1
Estimation of pantothenic acid liberated after treatment of enzymes A, B and C with alkali followed by alkaline phosphatase

Enzyme	Amino acid used for thioester binding	pmoles of enzyme bound amino acid	pmoles of pantothenic acid determined microbiologically
A	L-Ileu	206	135
	L-Leu	210	
B	L-Lys	167	0
	L-Orn	84	
C	L-His	100	70
	L-Phe	230	

The enzyme preparations used in this experiment are shown in fig.2. An aliquot of each enzyme preparation was used for the determination of the thioester binding of the substrate amino acids.

Fig.1. Fractionation of labelled bacitracin synthetase on the Sepharose column (see Materials and methods) into enzymes A, B and C. 0.5 ml of the 43–49% $(NH_4)_2SO_4$ fraction obtained from 5 g wet cells was put on a column (3 × 1.6 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.4, containing 0.2 mM $MgCl_2$ and 0.1 mM DTT (buffer B) at + 4°C. The column was eluted with buffer B (26 ml/h). After fraction No. 1, 0.1 M KCl was added to the buffer followed by a 0.1–0.4 M KCl gradient in buffer B at fraction No. 6. The volume of each fraction was 13 ml. The $^{32}PP_i$–ATP exchange activity dependent on L-ornithine (x-x-x), L-histidine (□-□-□) and L-cysteine (△-△-△) respectively was measured in a 0.5 ml aliquot of each fraction in order to locate the position of the three enzymes A (activates L-Ileu, L-Cys, L-Leu, L-Glu), B (activates L-Lys, L-Orn) and C (activates L-Ileu, L-Phe, L-His, L-Asp, L-Asn). For convenience the values for cysteine have been reduced by half. In addition aliquots of each fraction were used for the determination of [^{3}H]pantothenic acid (●-●-●).

degree of thioester-binding of the substrate amino acids in order to relate it to the amount of pantothenic acid found after release from the enzyme. It is seen (table 1) that enzymes A and C contain pantothenic acid and that the amount of pantothenic acid found is of the same order of magnitude as the amount of enzyme-bound substrate amino acids. However, no pantothenic acid was detected when using enzyme B. This is surprising in view of the incorporation of labelled pantothenic acid into enzyme B (fig.1). The negative outcome of this experiment was not due to any growth inhibitor substance of *L. plantarum* present in B, since when enzyme A was added enzyme B, the required amount of pantothenic acid was found in this mixture after treatment with alkali and phosphatase (results not shown). One possible explanation is that the pantothenic acid is bound differently in enzyme B to how it is in A and C and is therefore not liberated or destroyed during its liberation.

Recently Shimura et al. [10] reported the separation of bacitracin synthetase into three components I, II and III which presumably correspond to the three complementary enzymes A, B and C reported earlier by Frøyshov [7]. Using a microbiological method, they found pantothenic acid in all the fractions after treatment with alkali and phosphatase. However, the molar content of pantothenic acid in the enzyme, which corresponds to enzyme B, was considerably less than (less than 50%) that found in the others. The present work using a pantothenic acid-requiring organism demonstrates that pantothenic acid is part of all three enzymes. Furthermore, the liberation of pantothenic acid from enzyme A and C by alkali and phosphatase suggests that it in both enzymes pantothenic acid is bound in a similar manner as in gramicidin S synthetase [8]. However, from the present data it is doubtful whether pantothenic acid in enzyme B is bound in a similar manner.

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