COMPONENT I PROTEIN OF BACITRACIN SYNTHETASE: A MULTIFUNCTIONAL PROTEIN

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1. Introduction

Bacitracin synthetase has been shown to consist of 3 complementary components (components I, II and III [1], or enzymes A, B and C [2]). Component I activated L-isoleucine, L-cysteine, L-leucine, and L-glutamic acid in the presence of ATP and Mg^{2+}, and contained ~1 mol 4′-phosphopantetheine/mol component [1], suggesting the participation of component I in the synthesis of the straight peptide chain part of bacitracin molecule. The M_r of component I has been estimated to be ~200 000 by sucrose density gradient centrifugation [1] and 335 000 by gel filtration [3].

Attempts were made to obtain more exact information on the M_r and subunit structure of component I. The results obtained show that component I is a multifunctional protein composed of a single polypeptide chain with $M_r \sim 140\,000$, and that 5 discrete amino acid-activating enzymes are located on the single polypeptide chain.

2. Materials and methods

DEAE–Sephadex A-50 and Sephadex G-200 were obtained from Pharmacia Fine Chemicals AB. L-[^{14}C]Isoleucine (342 Ci/mol), L-[^{14}C]leucine (282 Ci/mol), and L-[^{14}C]glutamic acid (275 Ci/mol) were purchased from the Radiochemical Centre, and L-[^{14}C]cystine (295 Ci/mol) from New England Nuclear. BDH protein marker was obtained from BHD Chemicals. Minicon B 15 concentrators were obtained from Amicon Corp.

Abbreviations: PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; M_r, relative molecular mass

2.1. *Purification of component I*

Component I of bacitracin synthetase was purified from a crude lysate of *Bacillus licheniformis* by essentially the procedure in [4] except that the following two modifications were made to prevent proteolytic adgradation of the enzyme:

(i) Cells were washed 2 times each with 50 mM potassium phosphate buffer (pH 7.2) containing 0.15 M KCl and 50 mM potassium phosphate buffer (pH 7.2) containing 2 mM PMSF and 2 mM EDTA [5];
(ii) All the buffer solutions used in the purification steps were made in 0.2 mM PMSF and 0.25 mM EDTA.

The peak I fraction (16.7 mg protein) from hydroxyapatite column chromatography detailed in [4] was further purified by a DEAE–Sephadex A-50 column (1.5 × 30 cm) equilibrated with buffer A (10 mM potassium phosphate buffer (pH 7.2) containing 100 mM KCl, 0.2 mM PMSF, 0.25 mM EDTA, 1 mM 2-mercaptoethanol and 2% glycerol). After loading the sample to the column and washing the column with the same buffer, elution was carried out with a linear gradient containing 150 ml each of 0.25 M KCl and 0.5 M KCl in buffer A. Fractions of 6 ml were collected and assayed for amino acid-dependent ATP–PP_i exchange activity. Active fractions were pooled and used as purified component I throughout.

2.2. *Assay of ATP–$^{32}PP_i$ exchange activity*

Amino acid-dependent ATP–$^{32}PP_i$ exchange reaction was measured as in [4].

2.3. *Polyacrylamide gel electrophoresis*

This was done as in [6], using 5% separation gels

(0.5 × 8 cm, Tris–glycine buffer (pH 8.9)) and 2.5% stacking gels. Component I was pretreated with buffer A containing 25 mM DTT at 37°C for 12 h and subjected to PAGE. Electrophoresis was carried out at 5°C with a constant 2 mA/tube. Gels were stained for protein with Coomassie brilliant blue R-250, and additional gels were sliced into 2 mm segments and assayed for ATP–PP_i exchange activity.

2.4. *Preparation of ^{14}C-labelled amino acid-charged component I*

The reaction mixture contained the following in 0.2 ml: 10 μmol potassium phosphate buffer (pH 7.2), 2 μmol $MgCl_2$, 0.4 μmol ATP, 4 μmol DTT, 0.2 μCi ^{14}C-labelled amino acid and 96 μg component I protein preincubated in buffer A containing 25 mM DTT at 37°C for 12 h. After incubation at 37°C for 20 min, the reaction mixture was dialyzed at 4°C for 16 h against 10 mM sodium phosphate buffer (pH 7.0) containing 5 M urea, 25 mM DTT and 1% SDS. The dialyzed solution was concentrated with a Minicon B 15 concentrator to ~50 μl and subjected to SDS–urea–PAGE.

2.5. *SDS–Urea–polyacrylamide gel electrophoresis*

Gels (0.5 × 8 cm) contained 7.5% acrylamide, 0.2% methylene-bisacrylamide, 0.1% SDS, 2 M urea and 25 mM sodium phosphate buffer (pH 7.0). Samples were treated with buffer A containing 25 mM DTT at 37°C for 12 h, then heated at 100°C for 1 min (or incubated at 37°C for 12 h) in 25 mM sodium phosphate buffer (pH 7.0) containing 2 M urea and 1% SDS before application on gel. The gels were run at a constant 8 mA/tube for each with 25 mM sodium phosphate buffer (pH 7.0) containing 2 M urea and 0.1% SDS. After electrophoresis, gels were stained for protein with Coomassie brilliant blue R-250, and additional gels were sliced into 2 mm segments and assayed for radioactivity and pantothenic acid content. In measurement of radioactivity, gels were solubilized with 30% H_2O_2 at 60°C for several hours in tightly closed vials, and counted in 6 ml NT scintillator [7].

2.6. *Two-dimensional polyacrylamide gel electrophoresis*

The system used for two-dimensional gel electrophoresis combines the PAGE in [6] (see method 2.3) in the first with SDS–PAGE in the second dimensions. The first-dimensional PAGE was performed in 5% acrylamide rod (0.3 × 16 cm) and ~100 μg component I protein was applied on the gel. The gel was run at a constant 4 mA tube. At the end of the run, the gel was treated with 5% SDS as in [8]. Electrophoresis in the second dimension (24 × 16 × 0.3 cm) was carried out in 7.5% acrylamide gel at a constant 20 mA. After electrophoresis, gel was stained for protein with Coomassie brilliant blue R-250.

2.7. *Estimation of M_r-value of component I*

The M_r of component I was estimated by gel filtration on Sephadex G-200 and SDS–PAGE. In each experiment, component I was pretreated with buffer A containing 25 mM DTT at 37°C for 12 h. Gel filtration was performed on a 1.8 × 46 cm column with 10 mM potassium phosphate buffer (pH 7.2) containing 100 mM 2-mercaptoethanol at 4°C and 9 ml/h flow rate. A 2 ml sample containing 3–4 mg component I or protein markers was used.

SDS–PAGE was performed by the method in [9]. Gels (0.5 × 8 cm) containing 7.5% acrylamide were loaded with 24 μg component I protein or protein markers.

2.8. *Isoelectric focusing in polyacrylamide gel*

Isoelectric focusing was done as in [10,11], using 5% acrylamide gels (0.5 × 8 cm) containing 2% Ampholine (pH 3.5–10, LKB Instr.). The gels were loaded with 24 μg protein and run at 100 V for 6 h at 4°C. The anode electrolyte was 1 N NaOH and the cathode electrolyte was 0.02 M H_3PO_4.

2.9. *Determination of pantothenic acid*

Pantothenic acid was determined microbiologically with *Lactobacillus plantarum* ATCC 8014 as in [1].

2.10. *Protein assay*

Protein concentration was determined as in [12] with bovine serum albumin as a standard.

3. Results and discussion

When component I was purified using the buffers without added protease inhibitors, and was subjected to SDS–PAGE after overnight incubation at 37°C in the presence of SDS, a large number of protein bands were detected (not shown). This indicates that the enzyme preparation was contaminated with endogeneous proteases. Thus, to prevent proteolytic degradation of the enzyme, the buffers containing 0.2 mM

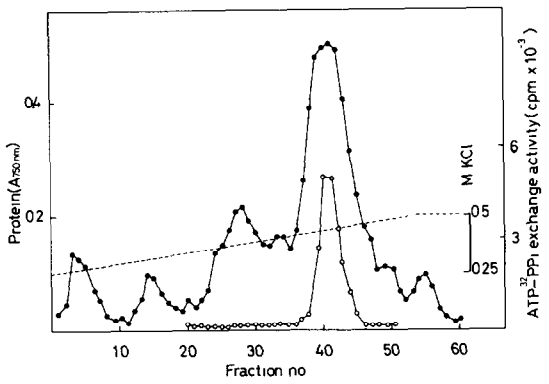
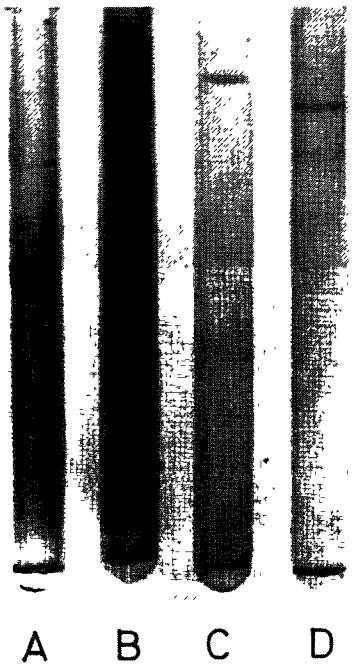


Fig.1. Purification of component I (a hydroxyapatite fraction) on DEAE–Sephadex A-50: (•) protein (absorbance at 750 nm); (○) ATP–PP_i exchange activity depending on L-cysteine.

PMSF and 0.25 mM EDTA were used throughout the purification procedures. These protease inhibitors completely suppressed the protease activity.

The column chromatographic profile on DEAE–Sephadex A-50 of the component I fraction obtained from a hydroxyapatite column is shown in fig.1. The fractions exhibiting the ATP–PP_i exchange activity dependent on L-cysteine were collected and subjected to PAGE in Tris–glycine buffer (pH 8.9). When the enzyme was pretreated with 25 mM DTT at 37°C for 12 h, a major single protein band was found (fig.2A)



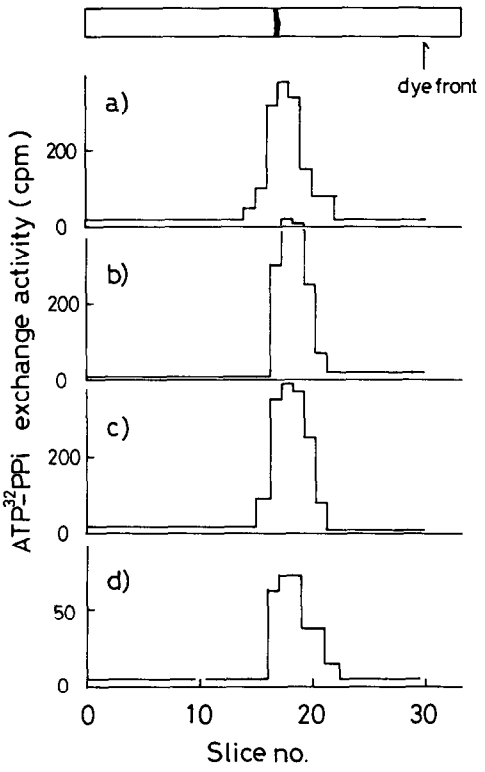


Fig.3. Polyacrylamide gel electrophoresis of component I in Tris–HCl glycine buffer (pH 8.9). The enzyme was subjected to gel electrophoresis under the same conditions as in fig.2A. After electrophoresis, gel slices were assayed for ATP–PP_i exchange activity depending on: (a) L-isoleucine; (b) L-cysteine (c) L-leucine; (d) L-glutamic acid.

and the peaks of ATP–PP_i exchange activities dependent on the 4 amino acids were coincident with the main band of enzyme protein (fig.3). On the other hand, when the enzyme was not pretreated with 25 mM DTT, an additional protein band with a slower mobility was found (fig.2B). Since this protein also activated the 4 amino acids (not shown) it is probably a dimer of component I. Similar results

Fig.2. Polyacrylamide gel electrophoresis of component I: (A) 5% acrylamide gel in Tris–glycine buffer (pH 8.9). The enzyme was incubated in 25 mM DTT at 37°C for 12 h; (B) The gel was the same as (A). The enzyme was not incubated; (C) 7.5% acrylamide gel in 25 mM phosphate buffer (pH 7.0) containing 0.1% SDS and 2 M urea. The enzyme was pretreated with 25 mM DTT at 37°C for 12 h, then heated with 1% SDS and 2 M urea at 100°C for 1 min (or incubated at 37°C for 12 h); (D) Isoelectric focusing in 5% acrylamide gel containing 2% carrier ampholite (pH 3.5–10).

showing the presence of monomer and dimer of the multienzyme of gramicidine *S*-synthetase were reported in [13].

To know whether component I is composed of one (or more) polypeptide chain(s), component I was subjected to SDS–urea–PAGE, isoelectric focusing, and two-dimensional PAGE as in section 2. As shown in fig.2C, 2D and 4, only one protein band was found and no splitting into subunits was observed. These results indicate that component I is composed of only one kind of polypeptide.

To obtain information on the number of polypeptide chains constituting component I, its M_r was estimated with native enzyme (gel filtration) and denatured one (SDS–PAGE). Fig.5 shows a plot of the logarithms of the M_r of several reference proteins *vs* their distribution coefficient on a Sephadex G-200 column. From the figure, the M_r of component I was estimated to be ~140 000. However, component I together with several reference marker proteins was

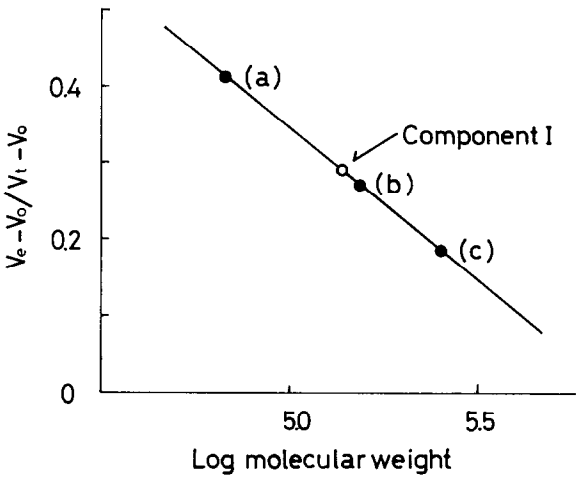


Fig.5. Estimation of M_r by gel filtration. Component I pretreated with 25 mM DTT or protein marker was loaded onto a Sephadex G-200 column (1.8 × 46 cm) equilibrated with 10 mM phosphate buffer (pH 7.2) containing 100 mM 2-mercaptoethanol. The protein markers used were: (a) bovine serum albumin (M_r 68 000); (b) aldolase (M_r 158 000); (c) catalase (M_r 250 000). V_e, V_o and V_t are elution volume, void volume and gel bed volume, respectively.

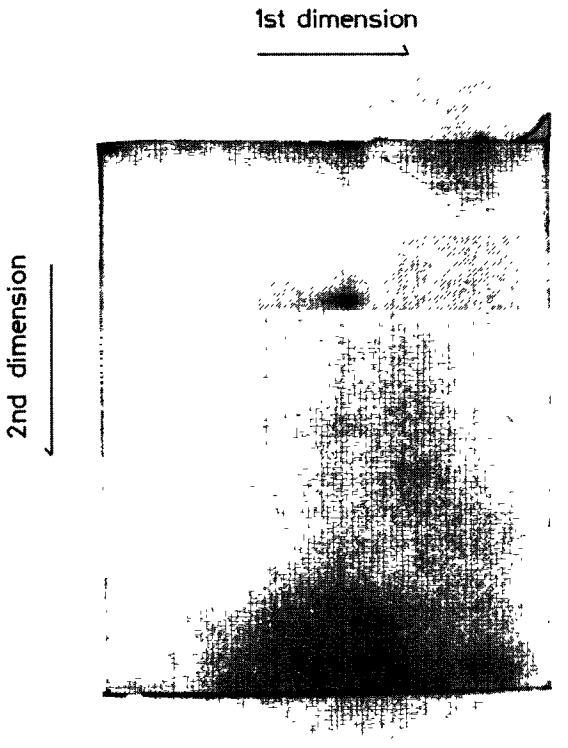


Fig.4. Two-dimensional gel electrophoresis of component I. The first dimension: 5% acrylamide gel in Tris–glycine buffer (pH 8.9). The second dimension: 7.5% acrylamide gel (24 × 16 × 0.3 cm) in 25 mM phosphate buffer (pH 7.0) containing 0.1% SDS.

subjected to SDS–PAGE and the relative mobilities of the reference proteins were plotted against the logarithms of their M_r as shown in fig.6. From the figure, the M_r of component I was calculated to be

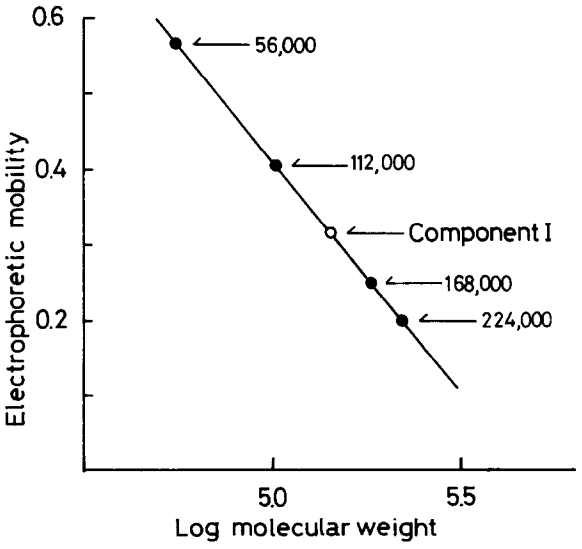


Fig.6. Estimation of M_r by SDS gel electrophoresis. 7.5% acrylamide gel in 25 mM phosphate buffer (pH 7.0) containing 0.1% SDS was used. BDH protein marker was used as standard M_r marker.

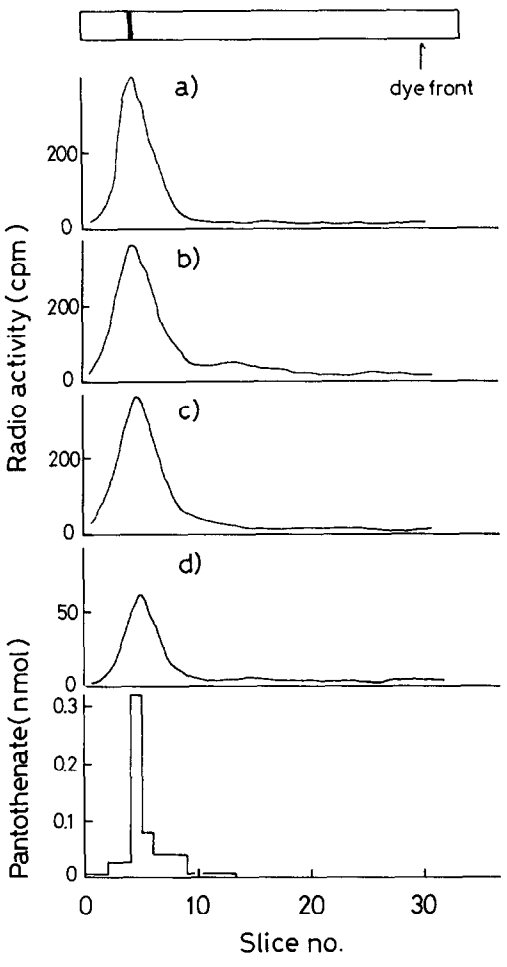


Fig.7. SDS–urea–gel electrophoresis of ^{14}C-labelled amino acid-charged component I. Component I was incubated with 0.2 μCi ^{14}C-labelled amino acid, 0.4 μmol ATP, and 2 μmol $MgCl_2$ in 0.2 ml at 37°C for 20 min. The reaction mixture was dialyzed against 10 mM phosphate buffer (pH 7.0) containing 1% SDS, 5 M urea, and 25 mM DTT, and subjected to gel electrophoresis exactly as in fig.2C. ^{14}C-labelled amino acids used were: (a) L-isoleucine; (b) L-cysteine; (c) L-leucine; (d) L-glutamic acid. Pantothenic acid was assayed microbiologically using *Lactobacillus plantarum* ATCC 8014.

~140 000 which is equal to that obtained by Sephadex G-200 gel filtration. These results indicate that component I consists of a single polypeptide chain having $M_r \sim 140\,000$. In [3] the M_r of purified enzyme A (component I) of bacitracin synthetase was reported as 335 000. As the M_r was estimated by gel filtration under a non-reduced condition [3], the value reported seems to correspond to that of a dimer of component I in our studies.

To prove the possibility that 5 amino acid-activating centers and phosphopantetheine arm are located on the single polypeptide chain, the component I protein pre-charged with labeled amino acids was subjected to SDS–urea–PAGE. As shown in fig.7, the peaks of the radioactivity of each amino acid and pantothenic acid content exactly coincided with the protein band. Since the activated amino acids are known to be bound to their specific sites on the enzyme with a thioester bond [14], this result indicates that component I is a multifunctional protein possessing 5 amino acid-activating centers and a phosphopantetheine arm. The multienzyme (heavy enzyme) of gramicidin *S*-synthetase has been shown to bear these properties [13,15].

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