THIAZOLINE RING FORMATION IN BACITRACIN BIOSYNTHESIS

Hiroaki ISHIHARA and Kensuke SHIMURA
Laboratory of Biochemistry, Department of Agricultural Chemistry, Tohoku University, Sendai 980, Japan

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1. Introduction

Bacitracin A contains a thiazoline ring in the N-terminal portion of the molecule. The thiazoline ring has been thought to be synthesized by a cyclic condensation of isoleucylcysteine portion of the peptide [1]. However, it has not been known when the thiazoline ring is formed in the process of bacitracin biosynthesis.

The work described here shows that the enzyme-bound dipeptide, a first intermediate peptide in peptide chain elongation in bacitracin biosynthesis [2], contained a thiazoline ring. This suggests that the thiazoline ring is formed at the first step of peptide chain elongation in bacitracin biosynthesis.

2. Materials

Thiopropyl–Sepharose 6B was obtained from Pharmacia Fine Chemicals AB, Uppsala. The gel was a mercaptohydroxypropyl-2-pyridyl disulfide derivative of Sepharose 6B and it contained about 20 μmol 2-pyridyl disulfide structure/ml swollen gel. Bacitracin (68.4 unit/mg) was obtained from Sigma Chemical Co., St Louis, MO.

3. Methods

3.1. *Preparation of bacitracin synthetase*

Bacitracin synthetase was partially purified as in [3], and the Sephadex G-200 fraction was used throughout the work.

3.2. *Preparation of bacitracin labeled with [^{14}C]-phenylalanine*

The reaction mixture (1.0 ml) with L-[^{14}C]phenylalanine was incubated for 3 h as in [3]. Carrier bacitracin (0.5 mg) was added and labeled bacitracin precipitated with saturated $(NH_4)_2SO_4$ was collected on a glass fiber filter as in [3]. The precipitate was dissolved in 4 ml methanol–water (1:1). After centrifugation for 10 min at 1000 × *g*, the supernatant was evaporated to dryness.

3.3. *Preparation of the isoleucylcysteine dipeptide fraction*

The reaction mixture contained the following in 0.8 ml final vol.: 40 μmol potassium phosphate (pH 7.2), 8 μmol $MgCl_2$, 1.6 μmol ATP, 8 μmol dithiothreitol, 0.2 μmol L-isoleucine, 0.2 μCi L-[^{14}C]-cystine (295 Ci/mol) and 2.1 mg protein of the enzyme. After incubation for 40 min at 37°C, the protein containing enzyme-bound dipeptide was precipitated with 4 ml cold 5% trichloroacetic acid (TCA). The precipitate was collected onto a glass fiber filter and washed 5 times with 5 ml portions of cold 5% TCA. The filter was dried in a stream of air at room temperature. The thin film of the precipitate was stripped off from the filter and dissolved in 2 ml 0.01 N KOH by stirring for 60 min at 20°C. The solution was incubated for 30 min at 50°C to liberate the covalently linked dipeptide. To the solution, 8 ml methanol was added and the mixture stored overnight at −10°C. The supernatant obtained by centrifugation was evaporated to dryness. The residue was dissolved in 0.2 ml water, then 3 ml methanol was added to the solution. After standing at 0°C for 30 min, the mixture was centrifuged at 1500 × *g* for 10 min. The

supernatant was evaporated to dryness.

In one experiment, 50 μl water was added to the residue, and the solution was applied to a silica gel thin-layer chromatogram which was developed in ethanol/28% NH_4OH/water (8:1:1). The dipeptide (R_F 0.67) was located by a chromatogram scanner, eluted with the same solvent, and concentrated to dryness.

3.4. *Opening of the thiazoline ring by acid hydrolysis*

The labeled bacitracin and the dipeptide fraction were heated in 0.5 N HCl for 10 min at 100°C or for 100 min at 60°C respectively, to open the thiazoline ring, [4]. When commercial bacitracin was used, 75–80% of the thiazoline ring was hydrolyzed under these conditions.

3.5. *Isolation of a thiol-containing peptide with thiopropyl–Sepharose 6B gel*

The residue containing the labeled bacitracin or the labeled dipeptide was dissolved in 1 ml water and was divided into two equal portions. One portion of the solution was heated in 0.5 N HCl as mentioned above to open the thiazoline ring, then adjusted to pH 7 with 0.5 N KOH. The other portion was treated similarly except that heating was omitted. To the solution a sufficient amount of dithiothreitol was added to reduce the thiol-containing peptide, and left for 10 min at room temperature. Thiopropyl–Sepharose 6B gel (1 ml) suspended in 3 ml 0.05 M potassium phosphate buffer (pH 7.2) containing 5 M urea, 2 mM Tris–HCl buffer (pH 7.2) and 1 mM EDTA was bubbled with nitrogen to exclude air and added to the sample solution. The gel suspension was gently shaken in an atomosphere of N_2 for 60 min at 30°C, then poured into a small column (4.5 × 100 mm). The eluate containing the non-attached peptide was collected. The column was washed with 25 ml 0.05 M phosphate buffer (pH 7.2) containing 1 mM EDTA, then with 15 ml 0.05 M phosphate buffer (pH 7.2) containing 1 M NaCl and 1 mM EDTA. The thiol-containing peptide was eluted with 20 mM 2-mercaptoethanol containing 50 mM phosphate buffer (pH 7.8) and 1 mM EDTA (attached fraction). All the eluates were collected in 2.6 ml each and 1 mM portion of the eluate was counted in a liquid scintillation spectrometer.

3.6. *Assay of antibiotic activity*

The antibacterial activity of bacitracin synthesized by the enzyme reaction was determined by an agar diffusion (paper-disc) method with *Micrococcus flavus* as test organism. The reaction mixture (2 ml) containing 2 mg protein of bacitracin synthetase, 2.5 mM each of the bacitracin-constituting amino acids and the other ingredients was incubated for 2.5 h as in [3]. After incubation, 8 ml methanol was added. The supernatant obtained by centrifugation was evaporated to dryness. The residue was extracted with methanol. The extract was evaporated to dryness and the residue was dissolved in 50 μl water. A paper disc was soaked in the solution and placed on an agar plate.

4. Results

4.1. *Experiments with the labeled bacitracin*

To know whether the bacitracin synthesized in the enzyme system contains a thiazoline ring in the molecule, [^{14}C]phenylalanine-labeled bacitracin was treated with thiopropyl–Sepharose 6B gel. Table 1 shows that 80% of the labeled bacitracin attached to the gel only after ring-opening treatment. This suggests that bacitracin synthesized by the in vitro system had a thiazoline ring.

Since the thiazoline ring has been thought to be essential for antimicrobial activity of bacitracin, antimicrobial activity of the products formed by the enzyme system was examined. As shown in table 2, an amount of apparent antibacterial activity was produced in the complete system. This fact supports the result mentioned above.

Table 1
Binding profile of ^{14}C-labeled bacitracin to thiopropyl–Sepharose 6B gel

Fraction	Radioactivity (dpm)	
	Non-treated	Acid-treated
Non-attached	53 080	7110
Attached	820	38 140

About 0.5 mg (50 320 dpm) of bacitracin was used in each experiment

Table 2
Formation of antimicrobial activity by the bacitracin synthetase

Incubation system	Bacitracin units	
	Expt. 1	Expt. 2
Complete (0 time)	0.25	0.48
Complete (2.5 h)	10	7.2
– all amino acids	0.25	–
– Asp and Asn	–	0.48

4.2. *Experiments with the labeled dipeptide*

Scan on the thin-layer chromatogram of the [^{14}C]-cysteine-labeled dipeptide fraction liberated from the enzyme protein by alkaline hydrolysis indicated that almost all the radioactivity was located in the position of the dipeptide, and that significant radioactivity was not detected in the positions of cystine and cysteine. Thus, the dipeptide fraction liberated by alkaline hydrolysis could be treated directly with thiopropyl–Sepharose gel without purification by thin-layer chromatography. Table 3 shows that the dipeptide has practically no free thiol groups, and the thiol group of the cysteine residue was liberated by the acid hydrolysis. These results suggest that the dipeptide contains a thiazoline ring.

To know whether the amino group of isoleucine residue of the dipeptide is free or blocked, [^{14}C]isoleucine-labeled dipeptide was oxidized by performic acid, and then analyzed by the dinitrofluorobenzene method [5]. About 70% of radioactivity was recovered as dinitrophenylisoleucine, indicating that the amino group of isoleucine residue was free.

5. Discussion

These results show that the thiazoline ring is formed at the first step of peptide chain elongation in bacitracin biosynthesis. This means that the other portion of the bacitracin molecule is not required for the thiazoline ring closure.

It was reported in the attempt at organic synthesis of the thiazoline ring that the cysteine residue could be cyclized to a thiazoline ring under anhydrous conditions only when the α-amino group of the N-terminal amino acid residue adjacent to the cysteine residue had been protected by an acetyl [6] or a benzyloxycarbonyl group [7]. It was shown in the present experiments that α-amino group of the isoleucine residue of the thiazoline ring-containing dipeptide was free. Thus, it is likely that the reaction of thiazoline ring formation in the present system is not spontaneous, but is catalyzed by an enzyme.

The amount of the attached dipeptide fraction decreased by leaving it at room temperature for a long time, or by the performance of thin-layer chromatography, before opening the thiazoline ring. This may be attributed to the change by oxidation in the structure of the thiazoline ring to a thiazole ring which is resistant to ring opening by heating in 0.5 N HCl [8].

Table 3
Binding profile of the [^{14}C]cysteine-labeled dipeptide to thiopropyl–Sepharose 6B gel

Fraction	Radioactivity (dpm)					
	Expt. 1		Expt. 2		Expt. 3	
	–	+	–	+	–	+
Non-attached	13 710	3390	13 320	5270	10 070	6060
Attached	420	10 900	260	7980	360	3640

In expts. 1 and 2, the dipeptide without purification by thin-layer chromatography (TLC) was used, and in expt. 3, it was purified by TLC. Radioactivities of the dipeptide used in each experiment in expts. 1,2 and 3 were 15 540 dpm, 15 320 dpm and 11 860 dpm, respectively. (–) not treated with 0.5 N HCl; (+) treated with 0.5 N HCl

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