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Further evidence for the presence of a thiazoline ring in the isoleucylcysteine dipeptide intermediate in bacitracin biosynthesis

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Isoleucylcysteine dipeptide, a first intermediate peptide in bacitracin biosynthesis, was liberated from the enzyme protein and oxidized with manganese dioxide in dimethylsulfoxide. The resulting oxidation product was identified by thin-layer chromatography as 2-(2-methyl-1-oxobutyl)-thiazole-4-carboxylic acid which has been isolated from the hydrolysate of bacitracin F. This result shows that the intermediate dipeptide contains a thiazoline ring, and that the thiazoline ring is synthesized at the dipeptide stage in the process of peptide chain elongation in bacitracin biosynthesis. Improbability of non-enzymatic dehydrative cyclization of the dipeptide is discussed.

Bacitracin; Thiazoline ring formation; Antibiotic biosynthesis

1. INTRODUCTION

Thiazoline and thiazole rings are found in several antibiotic polypeptides and have been thought to be synthesized by a dehydrative cyclization of cysteine-containing peptides [1]. However, the mechanism of biosynthesis of thiazoline ring has remained unsolved.

Bacitracin A, an antibiotic polypeptide produced by strains of *Bacillus licheniformis*, contains a thiazoline ring in the N-terminal portion of the molecule. One of the problems to be solved is when is the thiazoline ring formed in the process of peptide elongation in bacitracin biosynthesis. In our previous paper [2], it was suggested that the enzyme-bound isoleucylcysteine dipeptide, the first intermediate peptide in bacitracin biosynthesis [3], contains a thiazoline ring. The evidence for this came from the finding that the dipeptide attaches to a thiopropyl-Sepharose 6B gel by thiol-disulfide exchange, only when the dipeptide is treated with 0.5 N HCl at 60°C to open the ring structure and to liberate a free thiol group. In this paper, we present more direct evidence for the presence of a thiazoline ring in the dipeptide.

2. MATERIALS AND METHODS

2.1. *Chemicals*

The sources of materials used in this work were as follows: bacitracin (68.4 U/mg) from Sigma, L-[U-^{14}C]cysteine (spec. act. 295 Ci/mol) from

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NEN, and manganese dioxide from Junsei, used without any treatment for activation.

2.2. *Preparation of bacitracin synthetase*

Bacitracin synthetase was partially purified as described [4], and the Sephadex G-200 fraction was used throughout the work.

2.3. *Preparation of the '^{14}C-labeled dipeptide'*

The isoleucylcysteine dipeptide was prepared as described [2]. Briefly, the reaction mixture contained ATP, $MgCl_2$, L-isoleucine, L-[^{14}C]cysteine, dithiothreitol and the enzyme fraction. After incubation at 37°C for 20 min, protein was precipitated with 5% trichloroacetic acid, collected on a glass fiber filter, and washed with 5% trichloroacetic acid. The precipitate was dissolved in 0.01 N KOH and incubated at 50°C for 30 min to liberate the thioester-linked dipeptide. After removing protein by methanol precipitation, the supernatant was dried and used as the ^{14}C-labeled dipeptide.

Since the isoleucylcysteine dipeptide synthesized by the enzyme reaction is not simple L-Ile-L-Cys and has a ring structure as shown below, this compound will be referred to as the dipeptide or the ^{14}C-labeled dipeptide throughout this paper for the sake of simplicity.

2.4. *Opening the thiazoline ring by acid hydrolysis*

Peptides were treated in 0.5 N HCl at 100°C for 10 min or 60°C for 100 min to open the thiazoline ring, and then neutralized with 0.5 N KOH [2].

2.5. *Conversion of a cysteine-containing peptide to the disulfide compound by air oxidation*

To an aqueous solution of a cysteine-containing peptide (less than 1 μmol), NH_4OH and cupric sulfate were added to give final concentrations of 0.1 N and 0.01 mM, respectively. After standing at 37°C for 40 min, the solution was evaporated to dryness in vacuo.

2.6. *Preparation of bacitracin F from bacitracin A*

Bacitracin F was prepared by oxidation of bacitracin A with manganese dioxide (MnO_2) by a modification of the method of Barton et al. [5,6]. Bacitracin A is the main component of commercial bacitracin. MnO_2 (6 g) was added to a solution of bacitracin (600 mg) in dimethylsulfoxide (DMSO, 1.5 ml). The mixture was stirred for 3 days at room temperature. The course of the oxidation was monitored by the increase in absorption maximum at 290 nm due to the thiazole ring of bacitracin F. The supernatant obtained from the mixture by centrifugation at 8000 × *g* for 20 min was diluted 3-fold with water, and applied to an Amberlite CG-50 (H^+ form) column (1.3 × 10 cm). After washing the column with water, bacitracin F was eluted with 1 N HCl containing 50% methanol. The eluate was evaporated to dryness.

2.7. *Preparation of 2-(2-methyl-1-oxobutyl)-thiazole-4-carboxylic acid (I) from bacitracin F*

This compound was prepared according to Weisiger et al. [7]. Bacitracin F was hydrolyzed at 110°C for 18 h with 6 N HCl in a sealed tube. The hydrolysate was extracted three times with ethyl ether. The ether extract was concentrated and the product was crystallized from ethyl ether/petroleum ether. Yield: 10%; m.p. 94–95°C. Reported values: 94–95°C [7], 89.5–90°C [8]. Analysis calculated for $C_9H_{11}O_3NS$: C, 50.7; H, 5.22; N, 6.57; S, 15.0. Found: C, 50.1; H, 5.10; N, 6.57; S, 15.1.

2.8. *Oxidation of the dipeptide with manganese dioxide*

To the solution (0.5 ml) containing the ^{14}C-labeled dipeptide, 0.7 μmol of compound **I** and 0.35 μmol of bacitracin were added as carriers. The solution was evaporated to dryness in vacuo, and 3 mg of MnO_2 and 0.5 ml of DMSO were added to the residue. The mixture was shaken at 30°C for 4 days, diluted to 4 ml with water, and applied to a QAE-Sephadex (OH^- form) column (1 × 5 cm). After washing the column with water, the peptide was eluted with 1 N HCl. The eluate was extracted with ethyl ether. The ether layer was concentrated in vacuo. Aliquot samples were used for counting and the remainder was used for thin-layer chromatography.

2.9. *Thin-layer chromatography*

Thin-layer chromatography (TLC) was performed on Merck silica gel 60 plates (20 × 20 cm) with the following solvent system: (a) *n*-butanol/acetic acid/water (4:1:2, by vol.) and (b) chloroform/methanol/17% NH_4OH (16:15:5, by vol.).

Amino acids and peptides were detected with ninhydrin. Compound **I** was detected by UV absorption. Radioactivity was scanned by a chromatogram scanner.

2.10. *Synthesis of L-Ile-L-Cys*

L-Ile-L-Cys was synthesized according to Stoffel and Craig [9], crystallized as a free state from a 1 N HCl solution by adjusting the pH to about 4.8 with conc. NH_4OH, and recrystallized from water/ethanol.

3. RESULTS

3.1. *Analysis of the ^{14}C-labeled dipeptide by TLC*

To characterize the dipeptide, it was analysed by TLC. As shown in fig.1, a radioactive peak was detected near the area of L-isoleucine, but it did not coincide in position with either L-Ile-L-Cys or its air-oxidized product di-L-isoleucyl-L-cystine. On the other hand, when the dipeptide was treated with 0.5 N HCl at 60°C for 100 min, air-oxidized and subjected to TLC, a radioactive peak corresponding to di-L-isoleucyl L-cystine was observed (fig.2). These results show that the dipeptide is not simple L-Ile-L-Cys and can be converted to L-Ile-L-Cys by the ring-opening treatment with 0.5 N HCl, suggesting that the dipeptide contains a thiazoline ring.

3.2. *Identification of the dipeptide oxidized with MnO_2 as compound I*

The results described in [2] and the previous section clearly show that the thiol group of the cysteine residue in the dipeptide is not in a free state. However, this does not necessarily mean that a thiazoline ring is present in the dipeptide, because the thiol group may be blocked by some group (X group in fig.3) which can be removed by the ring-opening treatment. Therefore, to confirm more directly the presence of a thiazoline ring in the dipeptide, we tried to convert the dipeptide to a known compound of ring structure. If the dipeptide contains a thiazoline ring, a more stable thiazole derivative will be formed by oxidation with MnO_2 [5]. It might be expected that this thiazole derivative would be identical with compound **I**. This idea is schematically shown in fig.3. As shown below, this proved to be the case.

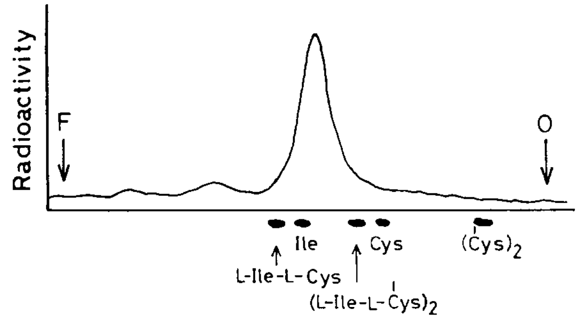

Fig.1. Thin-layer chromatography of the ^{14}C-labeled dipeptide. Solvent system, a; O, origin; F, solvent front.

The ^{14}C-labeled dipeptide was oxidized with MnO_2 in DMSO, applied to a QAE-Sephadex column, and eluted with 1 N HCl. The eluate was extracted with ethyl ether. 40–60% radioactivity used was recovered in the ether layer. The relatively low recovery of radioactivity in the ether layer would be due to the absence of a carrier having an identical structure. Solubility of the reaction product in the ether layer would be attributed to its aromatic ring structure. A sample of the ether layer was analysed by TLC. A radioactive peak coincided in position with compound **I** in the two solvent systems (fig.4).

These results show that the thiazole derivative derived from the dipeptide is identical to compound **I**. Therefore, the dipeptide liberated from the enzyme protein by mild alkali hydrolysis cer-

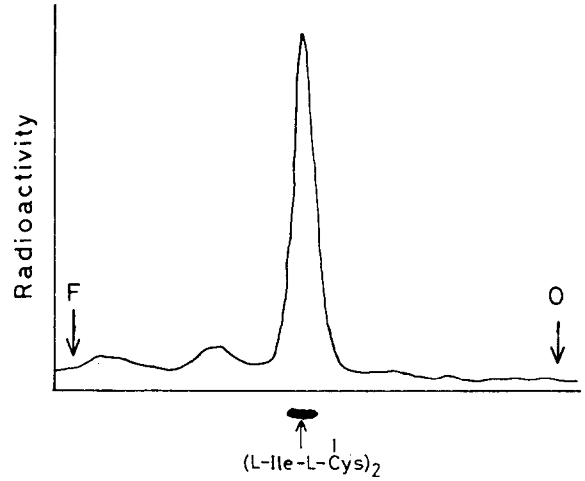

Fig.2. Thin-layer chromatography of the ^{14}C-labeled dipeptide which was treated with 0.5 N HCl and air-oxidized. Solvent system, a.

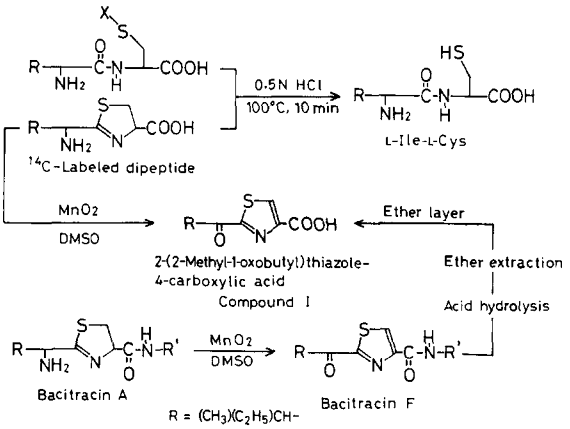

Fig.3. Scheme for identification of the ^{14}C-labeled dipeptide oxidized with MnO_2 as compound **I**.

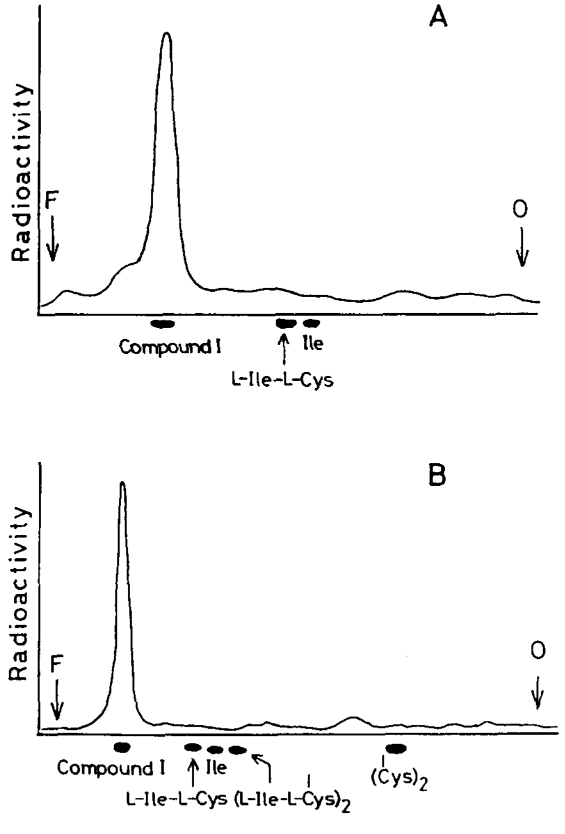

Fig.4. Thin-layer chromatography of the ^{14}C-labeled dipeptide which was oxidized with MnO_2 and extracted in the ether layer. (A) Solvent system a; (B) solvent system b.

tainly contains a thiazoline ring and its structure is probably 2-(1-amino-2-methylbutyl)-4,5-dihydrothiazole-4-carboxylic acid. Consequently, the ring structure must be formed at the dipeptide stage in the process of peptide elongation in bacitracin biosynthesis.

4. DISCUSSION

The results described above show that the intermediate dipeptide has a structure formed by a cyclic condensation of L-Ile-L-Cys. The following properties (Ishihara, H., unpublished) of the dipeptide also support this assumed structure having one amino and one carboxyl group. (i) It was bound to both Dowex 50 (H^+ form) and Dowex 1 (OH^- form). (ii) It migrated to the same position as neutral amino acids on paper electrophoresis at pH 6.8.

As discussed in our previous paper [2], it has been reported that cysteine-containing peptide could be non-enzymatically cyclized to a thiazoline ring, only when the α-amino group of the N-terminal amino acid adjacent to the cysteine residue had been protected [9,10]. Therefore, it is very unlikely that thiazoline ring formation in the dipeptide is non-enzymatic, and that ring closure occurred during the course of preparation of the dipeptide. To confirm this, L-Ile-L-Cys (0.5 mM) or 0.5 N HCl-treated bacitracin (thiazoline ring being opened, 0.18 mM), as a model compound, was left under the following conditions and ultraviolet absorption spectra were measured: (i) in 5% trichloroacetic acid at 4°C for 80 min; (ii) in 0.01 N KOH at room temperature for 60 min, then at 50°C for 40 min; (iii) in 95% methanol at room temperature for 12 h; and (iv) in DMSO at 30°C for 3 days. As expected, no change in spectra was observed under these conditions.

In preliminary experiments, we tried to separate the reaction of peptide bond formation from that of cyclization. The enzyme was treated with the following reagents for protein modification or under conditions giving 50–80% inhibition of the synthesis of enzyme-bound [^{14}C]cysteine-labeled dipeptide. Then the enzyme-bound product was liberated and assayed by TLC. These reagents and conditions were diethylpyrocarbonate, phenyloxazole, 2,3-butanedione, formaldehyde and sodium borohydride, potassium cyanate, high temperature, and acidic and alkaline pH values. In all cases analysed, only a radioactive peak corresponding to the dipeptide having a thiazoline ring was detected and no radioactive peak corresponding to L-Ile-L-Cys was observed. In some cases an additional peak corresponding to cysteine was detected. From these results, it seems likely that the two reactions are closely coupled and proceed at the same active site.

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REFERENCES

[1] Fowden, L. (1964) Annu. Rev. Biochem. 33, 173–204.
[2] Ishihara, H. and Shimura, K. (1979) FEBS Lett. 99, 109–112.
[3] Frøyshov, Ø. (1975) Eur. J. Biochem. 59, 201–206.
[4] Ishihara, H. and Shimura, K. (1974) Biochim. Biophys. Acta 338, 588–600.
[5] Barton, M.A., Kenner, G.W. and Sheppard, R.C. (1966) J. Chem. Soc. (C), 1061–1065.
[6] Barton, M.A., Kenner, G.W. and Sheppard, R.C. (1966) J. Chem. Soc. (C), 2115–2119.
[7] Weisiger, J.R., Hausmann, W. and Craig, L.C. (1955) J. Am. Chem. Soc. 77 3123–3127.
[8] Ariyoshi, Y., Shiba, T. and Kaneko, T. (1967) Bull. Chem. Soc. Jap. 40, 2648–2654.
[9] Stoffel, W. and Craig, L.C. (1961) J. Am. Chem. Soc. 83, 145–149.
[10] Hirotsu, Y., Shiba, T. and Kaneko, T. (1967) Bull. Chem. Soc. Jap. 40, 2950–2954.