Intracellular Colistin-like Active Substance of *Bacillus colistinus* and Interaction of Cellular Protein with Colistin†

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Received August 2, 1974

A colistin-like active substance was found in the cell extract of colistin-producing organism, *Bacillus colistinus*. This substance was designated colistin X and differed from known colistin on bioautograms and on gel filtration on Sephadex G-100 where it appeared in a protein fraction. Binding of ^{14}C -labeled colistin with the cellular protein of the organism was noted in low colistin ratios, such as approximately 260 u/mg protein, by incorporating radioactivity into the protein fraction in gel filtration. The paper radiogram of the binding reaction mixture gave a radioactive spot corresponding to colistin X. In contrast, in high colistin ratios the cellular protein used was partially degraded by reaction with colistin.

From these results, it was assumed that the intracellular colistin X was a binding complex of colistin and cellular protein.

Biosynthetic mechanisms of peptide antibiotics have been reported.^{1~8)} However, not much is known about the physiological role of peptide antibiotic within the cells of the producing organism.⁹⁾

Colistin, a peptide antibiotic produced by *Bacillus colistinus* KOYAMA, has been known as one of the polymyxin group antibiotics and identified with polymyxin E^{10~12)} as having a detergent-like action on sensitive bacteria.^{13~16)}

Colistin is formed by soluble fraction⁷⁾ and accumulated into the surrounding medium by growing cells of *B. colistinus*.¹⁷⁾ In order to obtain data on the intracellular behavior of the antibiotic, the activities and properties were investigated of a colistin-like active substance within the cells of the organism. The interaction of the cellular protein of the organism with colistin was also examined. This paper deals with the results obtained from these experiments.

MATERIALS AND METHODS

Bacterial strains and culture method. *Bacillus colistinus* KOYAMA was used as colistin-producing bacterium, and *Escherichia coli* NIHJ as test organism

† Physiology of Bacterial Production of Peptide Antibiotics. Part I.

for the assay of colistin activity.

B. colistinus was cultured in a synthetic medium which was composed of 10 g of soluble starch, 10 g of $(\text{NH}_4)_2\text{SO}_4$, 0.5 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g of NaCl, 10 μg of biotin and 2 g of CaCO_3 per liter (pH 7.0 ~ 7.2) at 30°C for 16 ~ 18 hr with shaking.

Extraction of intracellular colistin-like active substance from the cells of B. colistinus. Cells of the producing organism were harvested from 1 liter of the cultured medium, washed three times with Tris buffer composed of 10 mM Tris-hydroxymethylaminomethane, 2 mM MgSO_4 and 0.5 mM 2-mercaptoethanol (pH 7.2) and suspended in 15 ml of the same buffer. The suspension was sonicated at 10 kc for 20 min and centrifuged at 14,000 $\times g$ for 20 min. The resulting supernatant was used as the cell extract for intracellular colistin-like active substance.

Preparation of cellular protein of B. colistinus. Cells of the producing organism were harvested from 300 ml of the cultured medium, washed with and suspended in 15 ml of Tris buffer and subjected to sonication for 10 min. To the centrifugal supernatants of the sonicates was added $(\text{NH}_4)_2\text{SO}_4$ at a concentration of 100% saturation. The precipitates obtained from extracts equivalent to 1.2 liter of the cultured medium were dissolved in 7 ~ 10 ml of Tris buffer. To the solution was added $(\text{NH}_4)_2\text{SO}_4$ at a final concentration of 50% saturation and centrifuged. The supernatant was applied on a column of Sephadex G-100 (particle size 40 ~ 120 μ , equilibrated with Tris buffer, 1.5 $\times$ 42 cm), and eluted with Tris buffer. The fractions of the first

protein peak of eluates were combined and precipitated by the addition of $(\text{NH}_4)_2\text{SO}_4$ at a concentration of 80% saturation. The precipitates were collected and dissolved in 1.0~1.5 ml of Tris buffer. The gel filtration procedure was repeated once. The fractions of the main protein peak were collected, precipitated by $(\text{NH}_4)_2\text{SO}_4$ at 80% saturation and the precipitates were dissolved in 1 ml of Tris buffer and dialyzed against the same buffer. The dialyzed solution was used as the cellular protein.

Binding of colistin with the cellular protein of *B. colistinus*. The reaction was carried out in a mixture composed of 0.3 ml of the cellular protein prepared as described above (3.2 mg protein/ml in Tris buffer) and 50 μl of radioactive colistin (168,720 cpm and 5,021 u colistin/ml in water) in 0.35 ml of total volume, at 30°C for 5 min with shaking.

The incubated reaction mixture was examined on binding of colistin with protein by both gel filtration on Sephadex G-100 and paper chromatography. The amount of reaction mixture were 0.3 and 0.05 ml, respectively.

Gel filtration on Sephadex G-100. Samples of the cell extracts and incubated reaction mixtures were applied on a column of Sephadex G-100 (particle size 40~120 μ , equilibrated with Tris buffer, 1 $\times$ 18 cm) and eluted with Tris buffer at 3.7 ml/hr of flow rate. Fractionated eluates (1.4 ml/tube) were assayed for colistin activity or radioactivity and protein content determined.

Paper chromatography. An appropriate amount of sample was spotted on filter paper (Toyo-Roshi No. 50) and developed with a solvent system of *n*-butanol-acetic acid-water (3:1:1, by vol.) and/or *n*-butanol-acetic acid-pyridine-water (15:3:10:12, by vol.). Detection was carried out by color reaction (sprayed with 0.2% ninhydrin), antibacterial activity (bioautographed on *E. coli* plate) and radioactivity (assayed on filter paper strips cut every 0.05-Rf).

Preparation of radioactive colistin. Cultured medium containing ^{14}C -labeled colistin was obtained from aspartic acid- ^{14}C (U) (160 mCi/mmol, Daiichi Pure Chemicals Co. Ltd., Tokyo) using the method described by Ito *et al.*¹⁸⁾ The centrifugal supernatant of the cultured medium (77.7×10^6 dpm in 70 ml of total volume) was applied on a column of Amberlite IRC-50 (Na^+ form, 0.85×3.2 cm), washed with deionized water and eluted with 0.5 N-HCl-50% methanol. The eluted antibacterial fractions which contained radioactivity were combined, neutralized, dried by distillation *in vacuo* and dissolved in a solvent mixture composed of *n*-butanol, acetic acid, pyridine and water (15:3:10:12, by vol.). The solution was applied on a column of Sephadex LH-20 (0.9×45 cm) and eluted with the same solvent mixture. The fraction of radioactive and anti-

bacterial peak was repeatedly distilled *in vacuo* and dissolved in water freeing pyridine. The specific radioactivity of this preparation was 29.1 dpm/u colistin. The preparation gave only one spot having positive ninhydrin reaction, radioactivity and antibacterial activity on paper chromatograms using *n*-butanol-acetic acid-pyridine-water described above as the solvent system (Fig. 1). Furthermore, no ultraviolet absorp-

FIG. 1. Paper Chromatogram of Preparations of ^{14}C -labeled Colistin.

The fraction of radioactive peak eluted from Sephadex LH-20 of which radioactivity and colistin activity were 1897×10^3 cpm and 77×10^3 u/ml, respectively, was applied in 10- μl amount per spot and developed using the solvent *n*-butanol-acetic acid-pyridine-water (15:3:10:12). Detection was conducted by radioactivity (A), antibacterial activity (B) and ninhydrin reaction (C).

FIG. 2. Ultraviolet Absorption of Preparations of ^{14}C -labeled Colistin.

Labeled colistin preparation used in Fig. 1 was made free from pyridine and used at the concentration of 2000 u colistin/ml (●). The standard colistin was used as a control at the same concentration (○).

tion maximum was noticed that was similar to standard colistin (colistin sulfate, 18,500 u/mg, Kayaku Antibiotics Research Laboratory, Tokyo) as illustrated in Fig. 2.

Assay and determination. Protein content was determined spectrophotometrically at 280 nm using egg albumin (5×crystal, Miles Laboratories, Inc.) as the standard.

Colistin activity was assayed by pulp disk method on *E. coli* plates as described previously.¹⁷⁾

Radioactivity was measured with a Beckman LS-100 C liquid scintillation system. The scintillation fluid was composed of 1 part of Triton X-100 and 2 parts of 0.4% 2,5-diphenyloxazole-0.01% 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene-toluene. In radioactivity assay of filter paper strips of paper chromatograms, Triton X-100 was omitted from the scintillation fluid.

RESULTS

1. Intracellular colistin-like active substance of *B. colistinus*

The cell extract used contained 48 mg protein and 1,500 u colistin activity/ml. The extract was applied on a Sephadex G-100 column

FIG. 3. Gel Filtration on Sephadex G-100 of Intracellular Colistin-like Active Substance.

One ml of standard colistin (10,000 u/ml) (A) or of cell extract containing 1500 u colistin and 48 mg protein (B) was applied on the column (1×18 cm).

○—○, Colistin activity; ●—●, protein concentration.

and eluted with Tris buffer. The elution pattern is illustrated in Fig. 3. Colistin activity in the cell extract appeared as a peak at an earlier fraction (tube No. 5) concurrently with the main protein peak. In contrast under similar conditions, experiments using standard colistin indicated that colistin activity was fractionated at a later fraction (tube No. 10). In these experiments, protein and colistin activity in cell extract applied on the column were eluted at the recovery ratios of 106.9 and 99.9%, respectively and colistin activity in standard colistin applied was recovered 105.7% during gel filtration.

Bioautography of cell extract was also conducted. The results are shown in Fig. 4. In

FIG. 4. Bioautograms of Intracellular Colistin-like Active Substance.

Cell extract (CE) and standard colistin (St) used were described in Fig. 3. The solvents were *n*-butanol-acetic acid-water (3:1:1) (A) and *n*-butanol-acetic acid-pyridine-water (15:3:10:12) (B).

both solvent systems used, the cell extract appeared to contain an antibacterial substance which differed from known colistin in showing less migration from origin on paper chromatograms and in having somewhat turbid antibacterial spots on *E. coli* plates. This newly found component of intracellular colistin-like active substance is tentatively designated colistin X.

2. Change in intracellular colistin-like active substance during growth of *B. colistinus*

B. colistinus was cultured in the synthetic

medium and the appearance of colistin X in the cell extract followed. After 18-hr incubation, both cell growth and colistin activity attained maximal level. A low activity of antibiotic appeared in the cell extract before the maximal potency of extracellular antibiotic (Fig. 5A). Bioautogram of these cell extracts with the

FIG. 5. Time Course of Colistin X Emergence within the Cell of *B. colistinus*.

Bioautogram of cell extract (B) was conducted with the solvent *n*-butanol-acetic acid-water (3:1:1) on *E. coli* plates. ●—●, Optical density (O.D.) at 660nm after addition of 0.02 ml of 4 N HCl per ml of cultured medium; ○—○, colistin activity in supernatant of cultured medium; □—□, colistin activity in the cell extract.

lesser potency indicated that colistin X was temporarily present within the cells at a definite period, i.e., 14- to 16-hr culture, preceding the maximal potency in the surrounding medium (Fig. 5B). In the extract of 18-hr cultured cells, an antibacterial spot of which *R_f* was approximately 0.65 appeared, whereas colistin X was not detected. This higher *R_f* spot presumably corresponds to jolipeptin¹⁹⁾ according to Nakajima *et al.*²⁰⁾ On the other hand, in the centrifugal supernatants of cultured medium the known colistin appeared exclusively, whereas colistin X was not detected at any culture time so far tested.

3. Binding of radioactive colistin with cellular protein of *B. colistinus*

In a preliminary experiment using a crude protein preparation extracted from the cells of *B. colistinus* grown on glucose bouillon which did not produce colistin,¹⁸⁾ the reaction mixture

of colistin with this protein preparation gave antibacterial spots similar to colistin X-like spot on bioautography (Fig. 6), suggesting that

FIG. 6. Bioautogram of Colistin Incubated with Crude Protein Preparation Extracted from *B. colistinus*.

Standard colistin (800 u/ml) was incubated with a crude protein preparation (19.5 mg protein/ml) and bioautographed using *n*-butanol-acetic acid-water (3:1:1) as solvent and *E. coli* as test organism. The crude protein preparation used was extracted from glucose bouillon-grown cells of *B. colistinus* and subjected to streptomycin-treatment, fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ and dialysis. I, II: Incubated reaction mixture of standard colistin with crude protein preparation. III: Standard colistin alone.

colistin was bound with protein to form colistin X. To examine the binding of colistin with the cellular protein of the producing organism, experiments were carried out using radioactive colistin and the cellular protein as described under MATERIALS AND METHODS.

The incubated reaction mixture was applied on a column of Sephadex G-100 and eluted with Tris buffer. The elution pattern is shown in Fig. 7. On the identical gel filtration, the cellular protein indicated a single peak fractionated at tube No. 6, and the peak of radioactive colistin was at tube No. 10. The radio-

FIG. 7. Gel Filtration on Sephadex G-100 of ^{14}C -labeled Colistin Incubated with Cellular Protein.

Reaction mixture consisting of 0.3 ml of the cell protein (3.2 mg/ml) and 0.05 ml of ^{14}C -colistin (168720 cpm/ml) was incubated at 30°C , 5 min. ●—●, Radioactivity; □—□, protein concentration before reaction; ○—○, protein concentration after reaction with colistin.

activity in the incubated reaction mixture was fractionated significantly, in the protein fraction (tube No. 6), namely to the extent of approximately 5800 cpm/mg protein.

Paper chromatography of the incubated reac-

FIG. 8. Paper Radiogram of ^{14}C -labeled Colistin Incubated with Cellular Protein.

The incubated reaction mixture used was described in Fig. 7.

* Values represent relative radioactivity to the maximal peak when the radioactivity of the reaction mixture ($R_f 0.05 \sim 0.10$) and ^{14}C -colistin alone ($R_f 0.35 \sim 0.40$) were 618.9 and 251.4 cpm, respectively. The solvent used was *n*-butanol-acetic acid-water (3:1:1). —, Reaction mixture; —, ^{14}C -colistin alone.

tion mixture was also carried out. The results are shown in Fig. 8. The preparation of radioactive colistin alone gave the main spot in $R_f 0.35 \sim 0.40$ and a minor spot in $R_f 0.20 \sim 0.25$, whereas the reaction mixture gave a radioactive spot in $R_f 0.05 \sim 0.10$ which corresponded to colistin X in cell extracts of the producing organism.

From these results, it is likely that colistin binds with the cellular protein and the binding complex corresponds to colistin X which appears in the cell extracts of *B. colistinus*.

4. Dissociative effect of colistin on cellular protein of *B. colistinus*

In the reaction with radioactive colistin mentioned above (Fig. 7), it seemed that part of the protein was degraded as fractionated in later eluates. Detergents such as sodium dodecyl sulfate (SDS) are known to dissociate protein by reacting with the higher ratios of detergent/protein.²¹⁾ In order to determine the dissociative effect of colistin on protein,

FIG. 9. Gel Filtration on Sephadex G-100 of Cellular Protein Incubated with Higher Concentrations of Colistin.

Reaction mixture composed of 0.2 ml of protein (10.4 mg/ml) and 0.24 ml of colistin (18 mg/ml) was incubated at 30°C , 30 min. Aliquots of each fraction were analysed for protein concentration before (○—○), after (●—●) or without colistin (○—○) and for colistin activity after reaction (□—□).

experiments were carried out by using cold standard colistin at higher concentration. The results obtained with 2.4 mg colistin per mg of cellular protein are shown in Fig. 9. In the reaction mixture with colistin, an additional protein peak appeared in later fraction, such as tube No. 11 besides the main peak at tube No. 5, whereas samples before and after incubation with water instead of colistin gave the single peak fractionated in tube No. 5. From these results, it seems likely that protein is degraded in part by higher concentration of colistin, such as 2.4 mg per mg protein. Amount of the later eluted protein newly emerged in the reaction mixture with colistin corresponded to 33 per cent of the protein used. On the other hand, colistin activity in the reaction mixture appeared as a single peak at tube No. 10 and was not detected in the protein fraction, tube No. 5, indicating that no stable binding of colistin with protein was formed in the higher reaction ratios of colistin/protein.

DISCUSSION

Intracellular colistin-like active substance different from known colistin was detected and designated colistin X. It seems likely that colistin X may emerge rather as the binding product of already formed colistin with the cellular protein than as an intermediate of colistin biosynthesis, since it was assumed in this paper that binding product of colistin with the cellular protein corresponded to colistin X. On the other hand, several studies on the biosynthetic mechanism of peptide antibiotics have been reported.^{1~8)} In biosynthesis of gramicidin S, the elongation of the peptide chain with one-by-one addition of amino acids is involved but any high molecular precursor, such as colistin X may not be involved in peptide biosynthesis.^{1~4)}

It was noticed in this study that colistin bound with protein at lower concentrations of the antibiotic and degraded protein in higher concentrations of the antibiotic. The phenomenon is probably consistent with intracellular colistin activity increasing temporarily within the cell protein fraction in the form of

colistin X.

The interaction of colistin with protein could be explained presumably in terms of the detergent-like properties of colistin.^{13~16)} It is well known that detergents such as SDS bind primarily through hydrophobic forces to form complexes with protein in the diluted detergent solution^{22,23)} and dissociate protein when the ratio of detergent to protein is raised.²¹⁾ Interaction of detergents with protein was assumed to be relatively non-specific in a wide variety of proteins,^{22,23)} whereas certain proteins, such as thyroglobulin, are dissociated by SDS into sub-units;²¹⁾ others, such as human serum albumin form SDS complexes.²⁴⁾ From these facts and from a physiological point of view, specificity of colistin-protein interaction for cellular proteins of the producing organism, is of interest and under the investigation.

On the other hand, the mode of action of polymyxin group antibiotics against sensitive bacteria has been extensively investigated. It is assumed that antibiotics react with ionized phosphate groups of phospholipid components of the membrane^{13,14)} and that the antibiotics form insoluble complexes with RNA or DNA.^{25,26)} The possibility that interaction of colistin with these compounds could be involved in the formation of intracellular colistin X is not necessarily eliminated.

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