A NEW ENZYME IN *ESCHERICHIA COLI* CARRYING R-FACTOR PHOSPHORYLATING 3′-HYDROXYL OF BUTIROSIN A, KANAMYCIN, NEAMINE AND RIBOSTAMYCIN

Sir:

A new enzyme phosphorylating the 3′-hydroxyl group of butirosin A[1], kanamycin, neamine and ribostamycin (SF-733)[2] obtained from *Escherichia coli* carrying R-factor (JR 66/W 677). The enzyme was differentiated from the kanamycin-phosphorylating enzyme obtained from butirosin A-sensitive strains, *E. coli* K 12 ML 1629 and K 12 ML 1410 R 81.

In 1967, we reported[3~5] that kanamycin and paromamine were inactivated by an enzyme solution obtained from *E. coli* K 12 ML 1629 carrying R-factor. We also obtained a similar enzyme from *Pseudomonas aeruginosa* H 9 and the results of the studies on the structural requirements of the substrates and the inhibitors suggested the involvement of at least one of amino groups of deoxystreptamine moiety in binding with the enzyme[6,7].

Butirosin A, in which L-4-amino-2-hydroxybutyric acid is linked to the 1-NH_2 group of deoxystreptamine, inhibited *E. coli* K 12 ML 1629 or K 12 ML 1410 R 81 and was not inactivated by the enzyme solution prepared from these organisms. However, we found that the 100,000 *g* supernatant of disrupted cells of *E. coli* JR 66/W 677 phosphorylated the 3′-hydroxyl group of butirosin A. Thus, the two enzymes would appear to be different.

The cells of *E. coli* JR 66/W 677 were homogenized and the S-100 fraction, the supernatant of 100,000 *g* centrifugation, was prepared as described in a previous paper.[8] Butirosin A (100 mg) was inactivated at 37°C for 1 hour in a reaction mixture containing 12.5 mg of S-100 protein, 484.2 mg (800 μmoles) of disodium ATP in 5 ml of 0.1 N sodium bicarbonate, 20 ml of 1 M potassium phosphate buffer (pH 7.8) and 20 ml of 100 mM magnesium acetate in 60 mM 2-mercaptoethanol. The total volume was made 200 ml with distilled water. After the reaction, 200 ml of distilled water was added and the mixture kept in a boiling water bath for 10 minutes to stop the reaction. The solution was filtered and the filtrate was passed through a column of Amberlite CG-50 (NH_4^+ form, 50 ml). After washing the column with 1,000 ml of distilled water, the inactivated butirosin A was eluted with 0.1 N aqueous ammonia. The eluate which gave positive ninhydrin and RYDON-SMITH[9] reactions was collected and concentrated to dryness, yielding 101 mg of white powder. The powder was subjected to the rechromatography on Amberlite CG-50 (NH_4^+ form, 50 ml) and eluted with 0.05 N aqueous ammonia. Purified inactivated butirosin A (89 mg) was obtained as a white powder.

The inactivated butirosin A shows no antibiotic activity at 1 mg/ml by the cylinder-plate method using *Bacillus subtilis* PCI 219. It darkens at 217~221°C. Anal. calcd. for $C_{21}H_{42}N_5O_{15}P \cdot H_2O$: C 38.59, H 6.79, N 10.72, O 39.17, P 4.74. Found: C 38.11, H 6.85, N 10.20, O 38.38, P 4.44. It gives positive ninhydrin, RYDON-SMITH and HANES[10] reactions. On high-voltage paper electrophoresis under 3,500 volts for 15 minutes using formic acid - acetic acid - water (25 : 75 : 900 in volume), the inactivated butirosin A moves toward the cathode 14.6 cm, while butirosin A moves 19.0 cm. It shows only end absorption in the UV spectrum and a band at 960 cm^{-1} (phosphoric ester) in the IR spectrum. Hydrolysis of the inactivated butirosin A by alkaline phosphatase (*E. coli* origin; purchased from Boehringer Mannheim Co.), yields butirosin A. These properties indicate that the inactivated butirosin A is a monophosphate.

The pmr spectrum of the inactivated butirosin A in D_2O (20 mg/0.3 ml) using tetramethylsilane as an external reference ($\delta=0$) showed signals of the 4-amino-2-hydroxybutyric acid moiety at δ 4.78 (J=4.0 and 7.5 Hz, quartet, α-methine), δ 3.62 (J=7.5 Hz, triplet, γ-methylene) and *ca.* δ 2.5 (multiplet, β-methylene). The chemical shifts of these signals were the same as in butirosin A. Irradiation at δ 6.40 (1′-H) caused the signal at δ 3.62 (2′-H) to collapse to a doublet ($J_{2',3'}=10.0$ Hz). Irradiation at δ 3.62 indi-

Table 1. The heat stability of enzymes in S-100 fractions of *E. coli* K 12 ML 1629, *E. coli* K 12 ML 1410 R 81 and *E. coli* JR 66/W 677

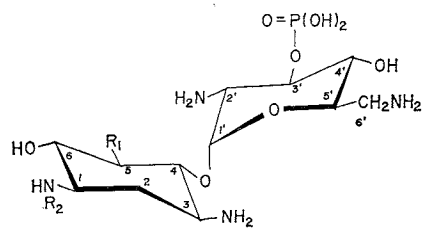
Strains	Antibiotics	Antibiotic inactivation (%)			
		none	45°C	55°C	65°C
E. coli K 12 ML 1629	Kanamycin	100	0	0	0
	Ribostamycin	100	10	0	0
	Butirosin A	0	0	0	0
E. coli K 12 ML 1410 R 81	Kanamycin	100	0	0	0
	Ribostamycin	100	20	0	0
	Butirosin A	0	0	0	0
E. coli JR 66/W 677	Kanamycin	100	39	5	10
	Ribostamycin	100	100	33	37
	Butirosin A	100	69	24	11

The S-100 fractions of three organisms were diluted with 20 mM potassium phosphate buffur (pH 7.8) to give the same degree of inactivation of kanamycin: *E. coli* K 12 ML 1629 and *E. coli* K 12 ML 1410 R 81, 10 mg protein/ml; *E. coli* JR 66/W 677, 50 μg protein/ml. The diluted enzyme solutions were heated for 5 minutes at the indicated temperature and were cooled to 37°C. The heated enzyme solution (0.2 ml) was added to the medium (0.8 ml) containing 2 μmoles of the antibiotic indicated, 160 μmoles ATP, 10 μmoles magnesium acetate, 6 μmoles 2-mercaptoethanol and 0.1 ml of 1 M potassium phosphate buffur (pH 7.8), and the reaction mixture was kept for 1 hour at 37°C. Antibiotic inactivation % was calculated from the residual antibiotic activity shown by the disc-plate method using *B. subtilis* PCI 219.

cated 3′-H signal at δ 4.5. The chemical shift of 3′-H was similar to that (δ 4.49) of 3′-H in ribostamycin 3′-phosphate. These observations indicated that the phosphoryl group was attached to the 3′-hydroxyl of 2,6-diamino-D-glucose moiety. It was further confirmed by the pmr study of the methanolysis product of the inactivated butirosin A.

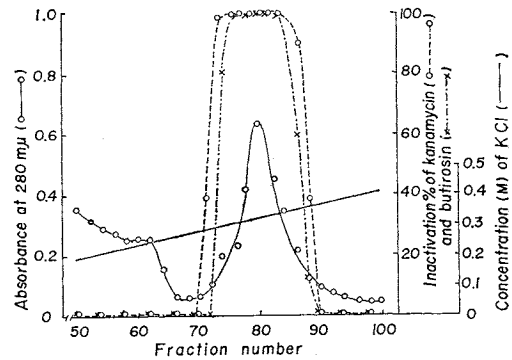
The inactivated butirosin A (41 mg) was refluxed with 0.4 N hydrogen chloride in methanol for 2 hours and a white powder (30 mg) of a methanolysis product was obtained by purification by Amberlite CG-50 (NH_4^+ form) column chromatography using 0.125 N aqueous ammonia. Refluxing of the methanolysis product with 2 N hydrogen chloride for 5 hours gave neamine, 4-amino-2-hydroxybutyric acid and phosphoric acid. The pmr spectrum of the methanolysis product (14.7 mg/0.3 ml) showed the signal of anomeric proton (1′-H) at δ 6.02. A spin decoupling experiment indicated the signals of 2′-H at δ 3.61 ($J_{1',2'}=3.5$ Hz, $J_{2',3'}=10.0$ Hz) and 3′-H at δ 4.66 ($J_{2',3'}=10.0$ Hz, $J_{3',4'}=9.0$ Hz, $J_{3',P}=8.5$ Hz). From the value of

Fig. 1. The structures of inactivated neamine, ribostamycin and butirosin A.



Neamine-3′-phosphate R_1 = OH, R_2 = H
Ribostamycin-3′-phosphate R_1 = β-D-Ribofuranosyl, R_2 = H
Butirosin A-3′-phosphate R_1 = β-D-Xylofuranosyl
R_2 = -CO·CH(OH)·CH_2·CH_2NH_2

Fig. 2. DEAE Sephadex A-50 column chromatography of the phosphorylating enzyme of *E. coli* JR 66/W 677.



the chemical shift and splitting pattern of 3′-H, the methanolysis product was determined to be N^1-(L-4-amino-2-hydroxybutyryl)neamine 3′-phosphate. Thus, it is conclusive that the inactivated butirosin A is butirosin A 3′-phosphate (Fig. 1).

Neamine, ribostamycin and kanamycin were inactivated by the S-100 fraction of *E. coli* JR 66/W 677 and the inactivated products were isolated by the procedure described above. They were confirmed by pmr analysis to be neamine 3′-phosphate, ribostamycin 3′-phosphate and kanamycin 3′-phosphate. The S-100 solutions prepared from *E. coli* K 12 ML 1629 and *E. coli* K 12 ML 1410 R 81 inactivated neamine, ribostamycin and kanamycin under the same conditions, yielding the 3′-phosphates of these antibiotics. However, these S-100 solutions did not inactivate butirosin A.

The enzyme in the S-100 fraction of *E. coli* JR 66/W 677 was different in the heat stability from those in the S-100 fraction of

E. coli K 12 ML 1629 and *E. coli* K 12 ML 1410 R 81 as shown in Table 1.

The enzyme in the S-100 fraction of *E. coli* JR 66/W 677 was precipitated by 30~50% saturation of ammonium sulfate and chromatographed on Sephadex G-100. The active fraction thus obtained was subjected to DEAE Sephadex A-50 column chromatography with a gradient of potassium chloride from 0 to 0.5 M. As shown in Fig. 2, the phosphorylating activity for both kanamycin and butirosin A appeared in the same fraction, indicating a single enzyme phosphorylating both antibiotics. Thus, the enzyme of *E. coli* JR 66/W 677 phosphorylating butirosin A was confirmed to be different from the enzymes of *E. coli* K 12 ML 1629 and *E. coli* K 12 ML 1410 R 81.

After finishing this study, we noted that BREZINSKA and DAVIES have reported the presence of two neomycin phosphotransferases I and II, in *E. coli* JR 39 and *E. coli* JR 66[11], respectively. It seems likely that the enzyme reported here is the same as DAVIES' neomycin phosphotransferase II.

Acknowledgement

We express our deep thanks to Prof. SUSUMU MITSUHASHI, Gunma University for his supply of *E. coli* K 12 ML 1410 R 81. We also thank Dr. SHUNTARO YASUDA, Meiji Seika Kaisha, Ltd., for his supply of ribostamycin and Dr. HIROSHI KAWAGUCHI, Bristol-Banyu Research Institute for the supply of butirosin A (Bu-1709 A_1).

MORIMASA YAGISAWA
HARUO YAMAMOTO
HIROSHI NAGANAWA
SHINICHI KONDO
TOMIO TAKEUCHI
HAMAO UMEZAWA

Institute of Microbial Chemistry,
Kamiosaki, Shinagawa-ku,
Tokyo, Japan

(Received October 23, 1972)

References

1) Woo, P.W.K.; H. W. DION & Q. R. BARTZ: Butirosins A and B, aminoglycoside antibiotics. III. Structures. Tetrahedron Letters 1971-26: 2625~2628, 1971
2) AKITA, E.; T. TSURUOKA, N. EZAKI & T. NIIDA: Studies on antibiotic SF-733, a new antibiotic. II. Chemical structure of antibiotic SF-733. J. Antibiotics 23: 173~183, 1970
3) UMEZAWA, H.; M. OKANISHI, S. KONDO, K. HAMANA, R. UTAHARA, K. MAEDA & S. MITSUHASHI: Phoshorylative inactivation of aminoglycosidic antibiotics by *Escherichia coli* carrying R-factor. Science 157: 1559~1561, 1967
4) OKANISHI, M.; S. KONDO, R. UTAHARA & H. UMEZAWA: Phosphorylation and inactivation of aminoglycosidic antibiotics by *E. coli* carrying R-factor. J. Antibiotics 21: 13~21, 1968
5) KONDO, S.; M. OKANISHI, R. UTAHARA, K. MAEDA & H. UMEZAWA: Isolation of kanamycin and paromamine inactivated by *E. coli* carrying R-factor. J. Antibiotics 21: 22~29, 1968
6) DOI, O.; S. KONDO, N. TANAKA & H. UMEZAWA: Purification and properties of kanamycin-phosphorylating enzyme from *Pseudomonas aeruginosa*. J. Antibiotics 22: 273~282, 1969
7) UMEZAWA, H.: Mechanisms of inactivation of aminoglycosidic antibiotics by enzymes of resistant organisms of clinical origin. Progress in Antimicrobial and Anticancer Chemotherapy (Proceedings of the 6th International Congress of Chemotherapy), Vol. 2; pp. 566~571, 1970. University of Tokyo Press.
8) YAGISAWA, M.; H. NAGANAWA, S. KONDO, M. HAMADA, T. TAKEUCHI & H. UMEZAWA: Adenylyldideoxykanamycin B, a product of the inactivation of dideoxykanamycin B by *Escherichia coli* carrying R-factor. J. Antibiotics 24: 911~912, 1971
9) RYDON, H. N. & P.W.G. SMITH: A new method for the detection of peptides and similar compounds on paper chromatograms. Nature 169: 922~923, 1952
10) HANES, C. S. & F. A. ISHERWOOD: Separation of the phosphoric esters on the filter paper chromatogram. Nature 164: 1107~1112, 1949
11) BRZEZINSKA, M. & J. DAVIES: Aminoglycoside-phosphorylating enzymes in neomycin and kanamycin resistant strains of *Escherichia coli* and *Pseudomonas aeruginosa*. Program and Abstracts, 12th Interscience Conference on Antimicrobial Agents and Chemotherapy, p. 84, 1972, American Society for Microbiology.