# LOCALIZATION OF SENSITIVITY TO KANAMYCIN AND STREPTOMYCIN IN 30 S RIBOSOMAL PROTEINS OF *ESCHERICHIA COLI*

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The localization in ribosomes of sensitivity to kanamycin was investigated and compared with that to streptomycin, using ribosomes from a mutant of *Escherichia coli* resistant to both these antibiotics. It was demonstrated that the sensitivity to both antibiotics was located in the 30 S subunit. The 30 S subunit was fractionated into a 23 S core and split proteins 30, and the 23 S core was split further into core proteins 30 and 16 S ribosomal RNA. It was found that the sensitivity to kanamycin as well as to streptomycin resided in proteins of the 23 S core. To identify the responsible component, 30 S ribosomal proteins from the parent strain and from a kanamycin-resistant mutant were labelled differently, and co-chromatographed through carboxymethylcellulose column. Only one component (P 10) was observed to appear inconsistently in the elution profile, and it was therefore assumed to be the component that determined the sensitivity to kanamycin. This assumption was confirmed by examining the inhibition by kanamycin in poly U-directed incorporation of phenylalanine with 30 S subunits reconstituted in different combinations between P 10 and the other components from the two origins. This component and the component controlling sensitivity to streptomycin were identical. It was observed that the reconstituted ribosomes containing P 10 from either resistant mutant were resistant to both antibiotics, and P 10 protein in both mutants was altered.

Aminoglycosidic antibiotics inhibit protein synthesis in bacteria and the site of their action is located in ribosomes[1,2]. Mutants resistant to these antibiotics are a very helpful tool for elucidation of the precise site and the mechanism of action of the antibiotics. Especially extensive studies have been carried out for this purpose using streptomycin (SM)-resistant mutants[3~10]. Recently, OZAKI, MIZUSHIMA and NOMURA showed that one component of 30 S ribosomal proteins was altered in SM-resistant mutants, and that this component was responsible for the resistance to SM in reconstitution experiment[11]. This result proved that mutation in the SM locus induces an alteration in the structure of a specific ribosomal protein to bring about resistance to SM.

Of the aminoglycosidic group, kanamycin (KM), SM, neomycin, paromomycin, gentamicin, hygromycin *etc.* have been observed to cause codon misreading[12,13,14]. Kasugamycin and spectinomycin, however, exhibit no misreading[15,16]. Although the mechanism of misreading is complicated[12,17], it seems that the deoxystreptamine or

streptamine moiety of these antibiotics plays an important role[18,19]. On the basis of chemical relationship, it can be expected that KM acts on ribosomes in a similar manner to SM. However, the resistance of microorganisms to the two antibiotics has a complicated correlation : *i. e.* some microorganisms show one-way cross-resistance, but others exhibit no significant cross-resistance[20,21]. The location of KM resistance and SM resistance in the same ribosomal protein would, of course, be less predictable if the ribosomes were not cross-resistant.

It is therefore interesting to elucidate the mechanism of action of KM, comparing with that of SM. In the present publication, it is demonstrated that one component of 30 S ribosomal proteins is altered in KM-resistant mutants, and that it is identical to the component determining sensitivity to SM.

## Materials

Organisms : *E. coli* strain NIHJ, a laboratory-developed KM-resistant mutant and a SM-resistant one were kindly given by the National Institute of Health, Tokyo, Japan. The minimal growth-inhibitory concentrations of KM and SM were both 2 $\mu$g/ml against the parent strain, 1,000 $\mu$g/ml and 64 $\mu$g/ml against the KM-resistant mutant, and 2 $\mu$g/ml and 3,000 $\mu$g/ml against the SM-resistant mutant, respectively. In polyphenylalanine synthesis with 70 S ribosomes from parent cell extract, percents inhibitions of KM and SM ($10^{-5}$ M) were 59 % and 46 %. Those of KM and SM were 0 % and 25 % against the KM-resistant ribosomes ; and 42 % and 2 % against the SM-resistant ribosomes. Therefore, KM-resistant mutant was resistant to SM, but SM-resistant one was sensitive to KM. The tendency of one-way cross resistance was observed in the protein synthesis, as in the case of antibacterial activity.

Chemicals : $^{14}$C-Phenylalanine (297 mC/mM), $^{14}$C-isoleucine (79.2 mC/mM), $^{14}$C-lysine (198 mC/mM) and $^{3}$H-lysine (256 mC/mM) were purchased from Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan. Polyuridylic acid and creatine phosphate were obtained from Calbiochem, Los Angeles, California, U. S. A.; guanosine-5′-triphosphate (GTP) and creatine phosphokinase from C. F. Boehringer & Soehne GmbH. Mannheim, Germany ; adenosine-5′-triphosphate (ATP) from Daiichi Pure Chemicals Co. Ltd.; cesium chloride from E. Merck AG Darmstadt, Germany. Carboxymethylcellulose (CMC, 0.77 meq/g) was the product of Brown Co. Ltd. Soluble RNA was prepared by phenol extraction from the 105,000 × $g$ supernatant fraction of *E. coli* NIHJ extracts[22].

## Methods

Preparation of Ribosomes and Ribosomal Subunits

The cells of *E. coli* NIHJ strain harvested at the late logarithmic phase of growth were ground with sea sand and extracted with standard buffer solution containing Mg acetate 10 mM, $NH_4Cl$ 50 mM, 2-mercaptoethanol 6 mM, and Tris-HCl 10 mM pH 7.6. The crude extract was centrifuged at 105,000 × $g$ for 2 hours to obtain ribosomes.

The preparation of 50 S and 30 S ribosomes, and 23 S core and split proteins 30 (SP30), and their reconstitution principally followed the mdthod of Hosokawa *et al.*[23] and Staehelin and Meselson[24].

Ribosomes were dissociated into 30 S and 50 S subunits by dialysis against standard buffer solution in which the concentration of Mg acetate was lowered to 0.1 mM. The suspension was layered on a sucrose gradient (5～20 %) and centrifuged in an SW 25 rotor (Beckmann) at 21,000 rpm for 14 hours at 4°C.

The 30 S subunits were further dissociated into 23 S core and SP 30 by centrifugation in 5.2 M cesium chloride solution in an SW 40 rotor (Beckmann) at 36,000 rpm for 38 hours.

The solution contained 40 mM Mg acetate and $2\times10^{-4}$ M EDTA[25]. SP 30 was recovered from the top of the gradient, and 23 S core was recovered from approximately at the middle of the gradient.

The preparation of 16 S ribosomal RNA and core proteins 30 (CP 30) from 23 S core particles followed the method of TRAUB and NOMURA[10]. The 16 S ribosomal RNA was prepared from the 23 S core by treatment with phenol. CP 30 from the 23 S core was prepared by the treatment with a solution consisted of 6 M urea, 2 M LiCl and 2.5 mM EDTA.

CMC Column Chromatography of 30 S Ribosomal Proteins

The 30 S ribosomal subunits prepared by the afore-mentioned procedure were dialysed against 2 M $NH_4Cl$, and then against 6 M urea containing 12 mM 2-mercaptoethanol. Ribosomal RNA in the sample was digested with 10 $\mu$g/ml each of pancreatic and $T_1$ RNases at 37°C for 20 minutes. The dissociated ribosomal proteins were applied on a CMC column, and eluted with a linear sodium acetate gradient (0.05~0.5 M) in the presence of 6 M urea solution containing 12 mM 2-mercaptoethanol. Protein concentration was determined by the method of LOWRY.

Labelling of Ribosomal Proteins

Ribosomal proteins were labelled with $^3$H- or $^{14}$C-lysine, principally following the method of ITOH, OTAKA and OSAWA[26,27]. Cells of *E. coli* NIHJ were grown aerobically for 4 hours at 37°C in Tris-NaCl (0.5 %)-glucose (0.3 %) medium supplemented with 10 $\mu$c/ml of $^3$H-lysine or 1 $\mu$c/ml of $^{14}$C-lysine, 50 $\mu$g/ml of L-methionine, and 20 $\mu$g/ml of each of other amino acids, and 300 $\mu$g/ml of yeast extract. Isotopically labelled 30 S or 50 S ribosomal proteins were prepared by the method described above.

In order to detect the alteration of ribosomal constituents, samples of 30 S or 50 S ribosomal proteins from $^3$H-lysine-labelled sensitive cells and from $^{14}$C-lysine-labelled resistant cells were mixed and simultaneously co-chromatographed on a CMC column. The radioactivity to each fraction was determined by a Packard liquid scintillation counter.

Reconstitution of Ribosomes from Ribosomal Proteins

Fractionated P 10 protein and the other ribosomal proteins from parent strain and resistant mutant were combined as described in Tables 5 and 6; and then after adding 16 S ribosomal RNA from the parent strain (weight ratio of ribosomal proteins/RNA=3), the mixture was dialysed against buffer solution containing 20 mM Mg acetate and 12 mM 2-mercaptoethanol. The reconstituted 30 S subunits were recovered by sucrose density gradient centrifugation. The 70 S ribosomes were obtained by adding 50 S subunits from the parent strain to the reconstituted 30 S subunits (50 S/30 S=2) and by dialysis and centrifugation.

## Results

### Effects of the Antibiotics on Reconstituted Ribosomes with 30 S and 50 S Subunits, and with 23 S Core, SP 30 and 50 S Subunits Derived from Parent Strain and Resistant Mutant

The 30 S and 50 S subunits from the parent strain and the KM-resistant mutant were reassociated in various combinations, and the poly U-directed incorporation of phenylalanine was assayed in the presence and absence of KM.

The incorporation of isoleucine by KM-induced miscoding in the presence of poly U was also examined. KM inhibited polyphenylalanine synthesis definitely when the ribosomes contained 30 S subunits derived from the parent strain, but much less when these subunits were derived from the resistant mutant. Whether 50 S subunits of the reassociated ribosomes were derived from parent strain or the resistant mutant did

not influence the inhibition by KM. Moreover, the poly U-directed incorporation of isoleucine in the presence of KM was more markedly stimulated with the ribosomes containing 30 S derived from the parent strain. These results show that the determinant of sensitivity to KM exists in 30 S subunits, but not in 50 S subunits.

The 23 S core and SP 30 were prepared from the two strains respectively, and 30 S subunits were reconstituted in different combinations. Not only inhibition of poly-

Table 1. Poly U-directed incorporation of phenylalanine and isoleucine by reconstituted ribosomes from *E. coli* NIHJ strain and the KM-resistant mutant.

| Ribosomal subunits | | | | Phenylalanine incorporated | | | | | Isoleucine incorporated | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 S | 30 S | 23 S | SP30 | Control | KM | (% Inhibition) | SM | (% Inhibition) | Control | KM | (% Stimulation) |
| S | S | | | 60.7 | 35.2 | (42) | 39.5 | (35) | 1.42 | 10.81 | (762) |
| S | R | | | 58.0 | 56.7 | ( 2) | 56.9 | ( 2) | 1.60 | 4.93 | (308) |
| R | S | | | 52.2 | 31.3 | (40) | 37.6 | (28) | 1.22 | 7.69 | (629) |
| R | R | | | 54.9 | 52.6 | ( 4) | 51.6 | ( 6) | 0.97 | 2.75 | (284) |
| S | | S | S | 47.2 | 28.3 | (40) | 32.6 | (31) | 1.65 | 8.50 | (515) |
| S | | S | R | 50.0 | 30.5 | (39) | 36.5 | (27) | 1.33 | 5.59 | (420) |
| S | | R | S | 45.6 | 44.8 | ( 2) | 46.1 | — | 1.36 | 3.07 | (226) |
| S | | R | R | 44.5 | 45.2 | — | 42.6 | ( 4) | 1.54 | 2.46 | (160) |
| R | | S | S | 40.1 | 26.0 | (35) | 26.9 | (33) | 1.08 | 4.26 | (394) |
| R | | S | R | 42.3 | 25.8 | (39) | 29.6 | (30) | 1.59 | 6.88 | (432) |
| R | | R | S | 38.0 | 36.8 | ( 3) | 35.3 | ( 7) | 0.86 | 1.79 | (208) |
| R | | R | R | 36.8 | 34.8 | ( 6) | 33.1 | (10) | 1.24 | 2.32 | (187) |

The number represents $\mu\mu$moles incorporated per mg of protein.
S : sensitive strain, R : resistant mutant, SP30 : split proteins of 30 S ribosomes, KM : kanamycin $2\times10^{-5}$ M, SM : streptomycin $2\times10^{-5}$ M.

Assay for incorporation was performed following the method of NIRENBERG and MATTHAEI[29]. The reaction mixture contained, in a volume of 0.50 ml, 50 S 230 $\mu$g, 30 S 120 $\mu$g, 23 S core 90 $\mu$g, SP30 80 $\mu$g, 105,000$\times g$ supernatant 150 $\mu$g, sRNA 150 $\mu$g, poly U 20 $\mu$g, ATP 1 $\mu$mole, GTP 0.05 $\mu$mole, creatine phosphate 2 $\mu$moles, creatine phosphokinase [EC 2.7.3.2] 50 $\mu$g, $^{14}$C-phenylalanine or $^{14}$C-isoleucine 0.1 $\mu$c, and kanamycin or streptomycin 0.01 $\mu$mole. The buffer solution used consisted of Mg acetate 10 mM, $NH_4Cl$ 50 mM, 2-mercaptoethanol 6 mM, and Tris-HCl 10 mM, pH 7.6.

It was incubated at 37℃ for 30 minutes. The radioactivity of hot TCA-insoluble fraction was determined by a windowless gas flow counter, and protein content by the method of LOWRY.

Table 2. Poly U-directed incorporation of phenylalanine and isoleucine by reconstituted ribosomes from *E. coli* NIHJ strain and the SM-resistant mutant.

| Ribosomal subunits | | | | Phenylalanine incorporated | | | | | Isoleucine incorporated | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 S | 30 S | 23 S | SP30 | Control | SM | (% Inhibition) | KM | (% Inhibition) | Control | SM | (% Stimulation) |
| S | S | | | 57.2 | 38.9 | (32) | 34.3 | (40) | 0.89 | 6.13 | (689) |
| S | R | | | 46.4 | 44.8 | ( 4) | 42.2 | ( 9) | 0.77 | 2.08 | (270) |
| R | S | | | 43.0 | 30.1 | (30) | 25.4 | (41) | 0.72 | 4.62 | (642) |
| R | R | | | 39.5 | 38.9 | ( 2) | 35.2 | (11) | 0.68 | 1.75 | (258) |
| S | | S | S | 40.3 | 28.6 | (29) | 25.8 | (36) | 0.65 | 2.76 | (425) |
| S | | S | R | 36.7 | 25.3 | (31) | 22.8 | (38) | 0.66 | 2.30 | (348) |
| S | | R | S | 38.9 | 37.0 | ( 5) | 35.8 | ( 8) | 0.58 | 0.97 | (167) |
| S | | R | R | 35.6 | 34.4 | ( 3) | 34.2 | ( 4) | 0.55 | 0.66 | (120) |
| R | | S | S | 30.8 | 22.1 | (28) | 19.4 | (37) | 0.61 | 1.74 | (285) |
| R | | S | R | 31.4 | 22.6 | (28) | 20.7 | (34) | 0.63 | 1.58 | (250) |
| R | | R | S | 27.5 | 26.6 | ( 3) | 24.8 | (10) | 0.59 | 0.62 | (105) |
| R | | R | R | 28.0 | 25.7 | ( 8) | 25.8 | ( 8) | 0.58 | 0.74 | (128) |

The reaction mixture and the assay conditions were the same as in Table 1.

peptide synthesis by KM, but also miscoding induced by KM was more markedly observed when the 23 S core of sensitive 30 S subunits was uesd. SP 30 did not affect the sensitivity to KM, irrespective of its origin. It can be concluded that the sensitivity to KM resides in 23 S core of 30 S subunits. The results are summarized in Table 1.

The same experiments were carried out as to the sensitivity to SM, using SM-resistant mutant. The results presented in Table 2 were quite similar to those on sensitivity to KM. Reassociated ribosomes derived from the KM-resistant mutant were resistant to SM (Table 1); similarly those derived from the SM-resistant mutant were resistant to KM (Table 2).

## Effects of Antibiotics on the Reconstituted Ribosomes with further Fractionated Components

The 16 S ribosomal RNA, CP 30 and SP 30 prepared from 30 S subunits of parent strain and resistant mutant were mixed in different combinations, and the mixtures

Table 3. Poly U-directed incorporation of phenylalanine and isoleucine by reconstituted ribosomes, using 16S ribosomal RNA, CP30 and SP30 from *E. coli* NIHJ strain and the KM-resistant mutant.

| Ribosomal subunits | | | Phenylalanine incorporated | | | | | Iosleucine incorporated | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 16S RNA | CP30 | SP30 | Control | KM | (% Inhibition) | SM | (% Inhibition) | Control | KM | (% Stimulation) |
| S | S | S | 44.9 | 26.9 | (40) | 31.0 | (31) | 1.14 | 6.43 | (564) |
| S | S | R | 41.7 | 26.2 | (37) | 27.9 | (33) | 1.01 | 4.73 | (468) |
| S | R | S | 42.3 | 37.7 | (11) | 38.9 | ( 8) | 0.95 | 2.09 | (220) |
| S | R | R | 40.0 | 37.2 | ( 7) | 35.2 | (12) | 1.08 | 1.89 | (179) |
| R | S | S | 42.5 | 27.2 | (36) | 31.0 | (27) | 1.21 | 5.38 | (447) |
| R | S | R | 43.5 | 28.3 | (35) | 32.6 | (25) | 0.95 | 3.67 | (386) |
| R | R | S | 39.1 | 41.2 | — | 37.1 | ( 5) | 0.89 | 1.39 | (157) |
| R | R | R | 39.4 | 37.0 | ( 6) | 38.2 | ( 3) | 0.83 | 1.14 | (138) |

The number represents $\mu\mu$moles incorporated per mg of protein.
S: sensitive strain, R: resistant mutant, KM: kanamycin $2\times10^{-5}$ M, SM: streptomycin $2\times10^{-5}$ M.
The reaction mixture, 0.50 ml, contained 50 S from parent strain 280 $\mu$g, SP30 85 $\mu$g, 16 S ribosomal RNA plus CP30 150 $\mu$g, 105,000$\times g$ supernatant 150 $\mu$g, sRNA 170 $\mu$g, poly U 20 $\mu$g, ATP 1 $\mu$mole, GTP 0.05 $\mu$mole, creatine phsphate 2 $\mu$moles, creatine phosphokinase [EC 2.7.3.2] 50 $\mu$g, $^{14}$C-phenylalanine or $^{14}$C-isoleucine 0.1 $\mu$c, and kanamycin or streptomycin 0.01 $\mu$mole.
The incorporation was assayed as described in Table 1.

Table 4. Poly U-directed incorporation of phenylalanine and isoleucine by reconstituted ribosomes, using 16S ribosomal RNA, CP30 and SP30 from *E. coli* NIHJ strain and the SM-resistant mutant.

| Ribosomal subunits | | | Phenylalanine incorporated | | | | | Isoleucine incorporated | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 16S RNA | CP30 | SP30 | Control | SM | (% Inhibition) | KM | (% Inhibition) | Control | SM | (% Stimulation) |
| S | S | S | 31.8 | 22.2 | (30) | 20.0 | (37) | 0.65 | 2.08 | (320) |
| S | S | R | 28.4 | 20.4 | (28) | 16.8 | (41) | 0.71 | 2.18 | (307) |
| S | R | S | 25.0 | 22.8 | ( 9) | 22.5 | (10) | 0.68 | 0.76 | (112) |
| S | R | R | 25.3 | 24.2 | ( 4) | 23.3 | ( 8) | 0.62 | 0.59 | ( 95) |
| R | S | S | 21.5 | 16.1 | (25) | 14.2 | (34) | 0.70 | 1.64 | (234) |
| R | S | R | 18.2 | 12.9 | (29) | 12.0 | (34) | 0.65 | 1.67 | (257) |
| R | R | S | 20.1 | 18.9 | ( 6) | 17.9 | (11) | 0.58 | 0.63 | (109) |
| R | R | R | 19.8 | 19.2 | ( 3) | 18.4 | ( 7) | 0.56 | 0.63 | (112) |

The reaction mixture was the same as described in Table 3.

were dialysed against a buffer solution containing 10 mM Mg acetate. The reconstituted 30 S particles were recovered by centrifugation. The 50 S subunits derived from the parent strain were added to obtain 70 S ribosomes, whose sensitivities to KM and SM were investigated.

KM and SM inhibited in a similar degree the poly U-directed incorporation of phenylalanine, and also stimulated markedly the incorporation of isoleucine when the reconstituted 30 S subunits were composed of CP 30 derived from the parent strain. But the two antibiotics did not inhibit the poly U-directed incorporation of phenyl-

Fig. 1. Co-chromatography on carboxymethyl-cellulose (CMC) columns of labelled ribosomal proteins from parent strain and KM-resistant mutant

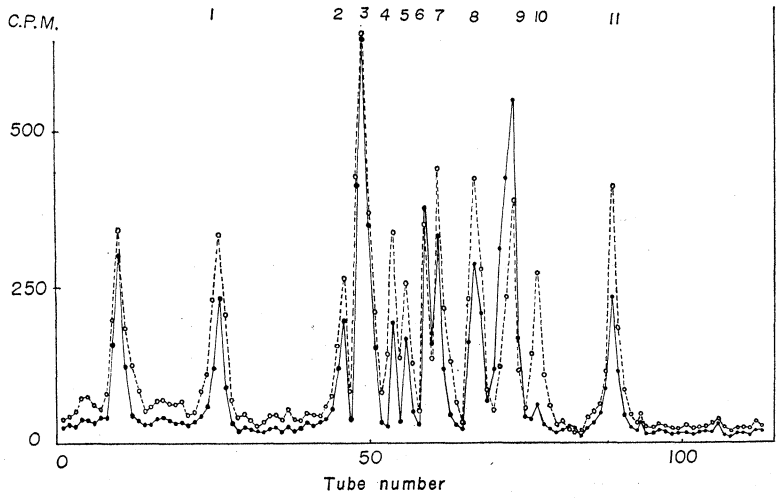
---------: $^{3}H$-lysine-labelled protein of parent strain

———: $^{14}C$-lysine-labelled protein of KM-resistant mutant

Sample of about 8 mg was placed on a CMC column (size 0.8×60 cm). Proteins were eluted from the column with a linear sodium acetate gradient (0.05~0.5 M). The flow rate was 1.5 ml per 12 minutes.

To measure the double-labelled radioactivity in proteins, carrier of serum albumin was added to each fraction; and the radioactivity of the cold TCA-insoluble fraction was determined by a Packard liquid scintillation counter.

(a) 30S protein




(b) 50S protein

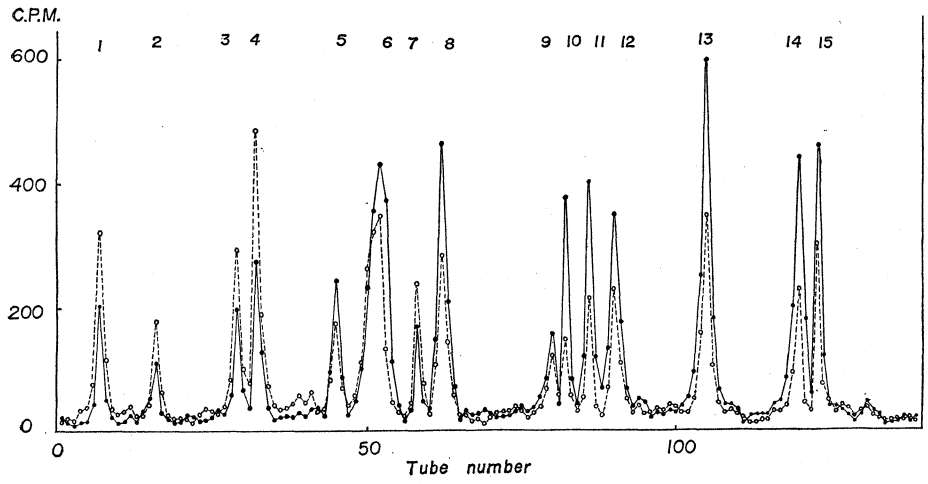
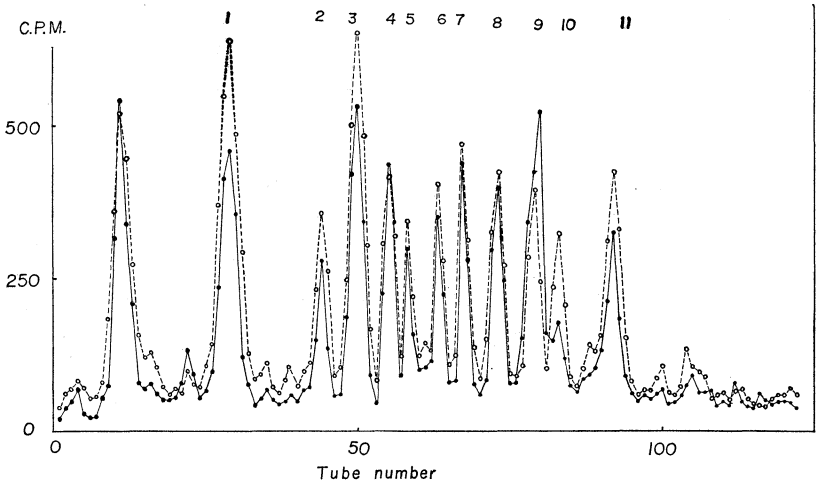


Fig. 2. Co-chromatography on CMC columns of labelled ribosomal proteins from parent strain and SM-resistant mutant

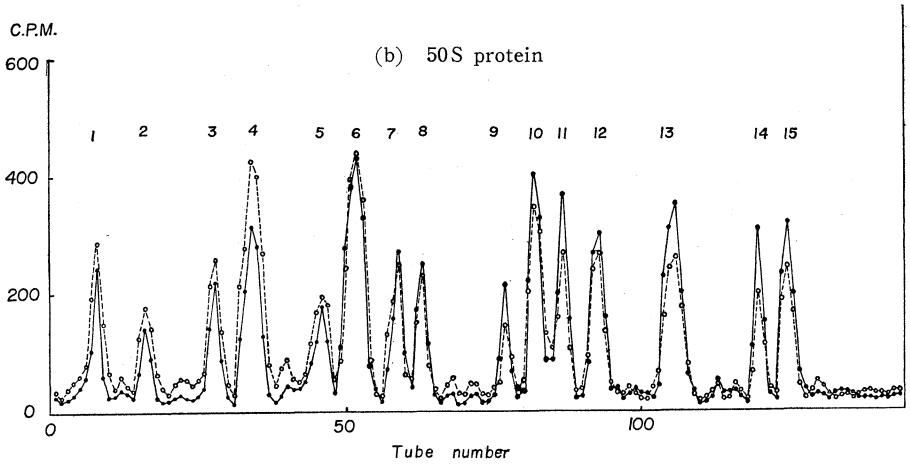
·····: $^{3}$H-lysine-labelled protein of parent strain

———: $^{14}$C-lysine-labelled protein of SM-resistant mutant

(a) 30S protein




(b) 50S protein




alanine, nor stimulate the incorporation of isoleucine, when the system contained the CP 30 derived from the mutant resistant to the corresponding antibiotic. The results are shown in Tables 3 and 4.

The origin of SP 30 and 16S ribosomal RNA, in contrast, did not affect the sensitivity of the reconstituted 30S subunits.

The present investigation indicates that the alteration which causes KM resistance and SM resistance is in CP 30 of the mutants.

Difference in Ribosomal Proteins between Parent Strain and Resistant Mutant

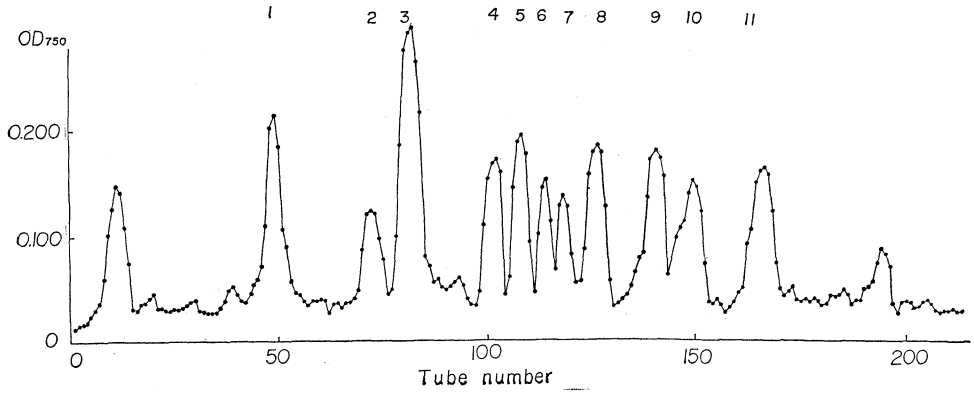
The 30S or 50S ribosomal proteins of parent strain were labelled with $^{3}$H-lysine, and those of KM-resistant mutant were labelled with $^{14}$C-lysine, as described in "Method". The dissociated 30S ribosomal proteins from the two origins, were mixed and chromatographed with a CMC column to detect any difference in the proteins of the parent strain and the resistant mutant. As shown in Fig. 1a, the peak 9 of

Fig. 3. Chromatography of 30 S ribosomal proteins from parent strain and those from KM-resistant mutant

(a) and (b): Sample of about 180 mg was applied on a CMC column (size 1.9×95 cm). Proteins were eluted from the column with a linear sodium acetate gradient (0.05~0.5 M). The flow rate was 1.5 ml per 15 minutes. Protein content was determined by the method of LOWRY.

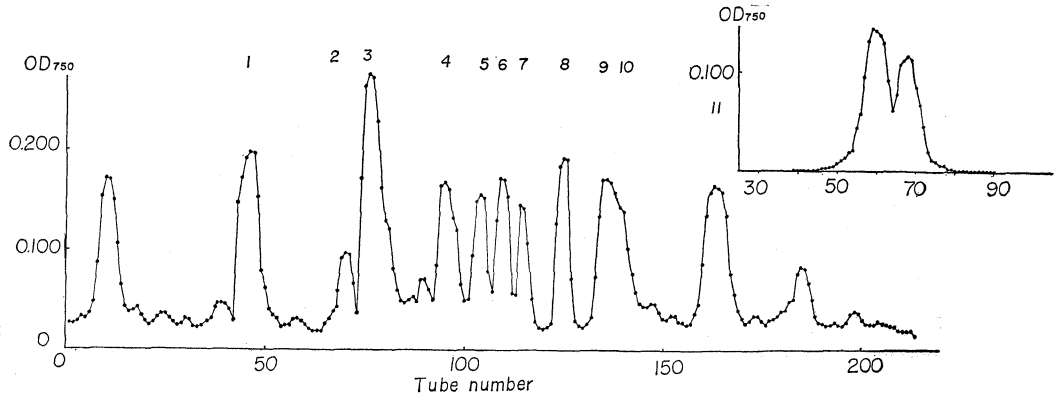
(c): Fractions containing No. 9 and No. 10 components from KM-resistant mutant were pooled and applied to another column (size 1.9×42 cm). Elution was done with a linear sodium acetate gradient (0.2~0.6 M).

(a) Proteins from parent strain




(b) Proteins from KM-resistant mutant

(c) Rechromatography of components from KM-resistant mutant




$^{14}$C-lysine was remarkably higher relative to the corresponding peak of $^{3}$H-lysine, and the peak 10 of $^{14}$C-lysine was hardly detected. The other peaks of $^{14}$C- and $^{3}$H-lysine were located at identical positions. The SM-resistant mutant resembled the KM-resistant mutant (Fig. 2 a).

The fractionation of 50 S ribosomal proteins was performed in the same method as described above. No significant difference was observed between the parent strain and either mutant (Figs. 1 b, 2 b).

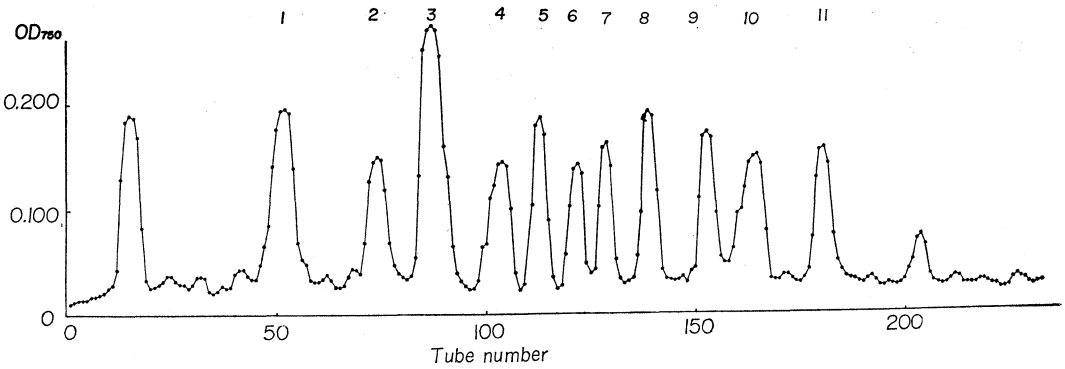
When the fraction constituting peak 9 from either resistant 30 S subunit was re-chromatographed with a CMC column, two peaks were separated from each other (Figs. 3 c and 4 c). It thus appears that either mutation caused protein P 10 to move more slowly on CMC.

Effects of Antibiotics on 30 S Subunits Reconstituted by Combining Protein P 10 and Complementary Ribosomal Proteins from Parent Strain and Resistant Mutant

Fig. 4. Chromatography of 30 S ribosomal proteins from parent strain and those from SM-resistant mutant

The procedure was done as described in Fig. 3.

(a) Proteins from parent strain




(b) Proteins from SM-resistant mutant

(c) Rechromatography of components from SM-resistant mutant

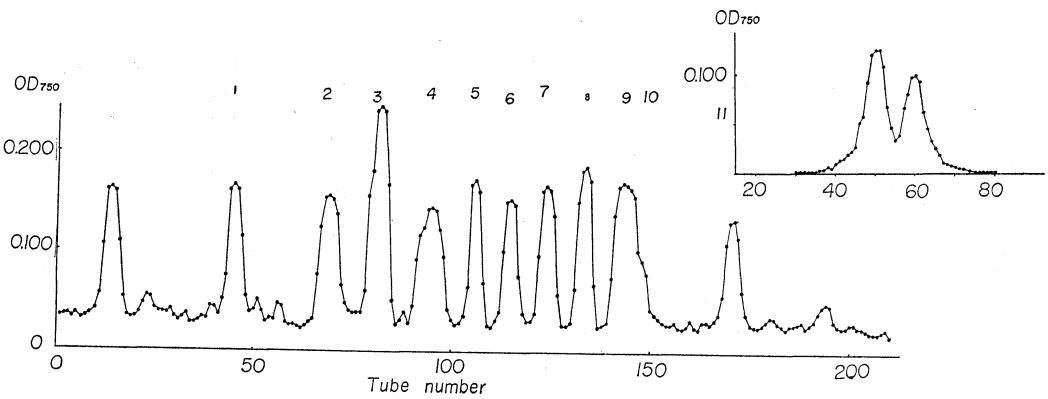



Table 5. Poly U-directed incorporation of phenylalanine and isoleucine by reconstituted ribosomes, using 30S ribosomal proteins from *E. coli* NIHJ strain and the KM-resistant mutant.

| Ribosomal proteins | | Phenylalanine incorporated | | | | | Isoleucine incorporated | | |
|---|---|---|---|---|---|---|---|---|---|
| ∑Pi-P10 | P10 | Control | KM | (% Inhibition) | SM | (% Inhibition) | Control | KM | (% Stimulation) |
| S | S | 10.24 | 6.34 | (38) | 7.30 | (29) | 0.25 | 1.85 | (740) |
| S | R | 9.07 | 8.34 | ( 8) | 7.98 | (12) | 0.21 | 0.44 | (209) |
| S | — | 2.70 | 2.54 | ( 6) | 2.59 | ( 4) | 0.18 | 0.75 | (416) |
| R | S | 6.88 | 5.01 | (27) | 5.42 | (21) | 0.17 | 0.79 | (465) |
| R | R | 6.02 | 5.48 | ( 9) | 5.66 | ( 6) | 0.14 | 0.17 | (121) |
| R | — | 2.24 | 2.19 | ( 2) | 2.13 | ( 5) | 0.10 | 0.24 | (240) |
| 30 S (S) | | 20.95 | 11.10 | (47) | 13.61 | (35) | 0.62 | 7.24 | (1,168) |
| 30 S (R) | | 15.77 | 15.30 | ( 3) | 14.95 | ( 5) | 0.58 | 2.07 | (358) |

The number represents $\mu\mu$moles incorporated per mg of protein.

S: sensitive strain, R: resistant mutant, KM: kanamycin $2\times10^{-5}$ M, SM: streptomycin $2\times10^{-5}$ M, P10: peak 10 ribosomal protein, ∑Pi-P10: all other proteins except P10.

The reaction mixture contained, in a volume of 0.50 ml, 50 S from parent strain 200 $\mu$g, reconstituted 30 S subunit consisting of ribosomal proteins in different combination 105 $\mu$g, 105,000×$g$ supernatant 120 $\mu$g, sRNA 100 $\mu$g, poly U 20 $\mu$g, ATP 1 $\mu$mole, GTP 0.05 $\mu$mole, creatine phosphate 2 $\mu$moles, creatine phosphokinase 50 $\mu$g, $^{14}$C-phenylalanine or $^{14}$C-isoleucine 0.1 $\mu$c, and kanamycin or streptomycin 0.01 $\mu$mole.

The buffer solution used consisted of Mg acetate 10 mM, $NH_4Cl$ 50 mM, 2-mercaptoethanol 12 mM, and Tris-HCl 10 mM, pH 7.6. The incorporation was assayed as described in Table 1.

Table 6. Poly U-directed incorporation of phenylalanine and isoleucine by reconstituted ribosomes, using 30S ribosomal proteins from *E. coli* NIHJ strain and the SM-resistant mutant.

| Ribosomal proteins | | Phenylalanine incorporated | | | | | Isoleucine incorporated | | |
|---|---|---|---|---|---|---|---|---|---|
| ∑Pi-P10 | P10 | Control | SM | (% Inhibition) | KM | (% Inhibition) | Control | SM | (% Stimulation) |
| S | S | 7.12 | 5.13 | (28) | 4.85 | (32) | 0.42 | 2.75 | (655) |
| S | R | 6.64 | 5.98 | (10) | 5.58 | (16) | 0.38 | 0.82 | (216) |
| S | — | 2.35 | 2.21 | ( 6) | 2.14 | ( 9) | 0.28 | 1.04 | (371) |
| R | S | 5.18 | 3.88 | (25) | 3.72 | (28) | 0.18 | 0.99 | (550) |
| R | R | 6.03 | 5.85 | ( 3) | 5.25 | (13) | 0.20 | 0.36 | (180) |
| R | — | 1.78 | 1.71 | ( 4) | 1.66 | ( 7) | 0.16 | 0.41 | (256) |
| 30S (S) | | 18.11 | 11.24 | (38) | 10.89 | (40) | 0.83 | 8.12 | (980) |
| 30S (R) | | 13.89 | 13.20 | ( 5) | 12.21 | (12) | 0.62 | 2.23 | (360) |

The reaction mixture was simliar to that described in Table 5.

The 30S ribosomal proteins from parent strain and KM- or SM-resistant mutant were fractionated by CMC column chromatography. The 30S subunits were reconstituted, as described in "Methods", from P10 and the other 30S ribosomal proteins from the parent strain and resistant mutants in various combinations. The 16S ribosomal RNA and 50S subunits used were prepared from parent strain. As shown in Tables 5 and 6, KM and SM inhibited the poly U-directed incorporation of phenylalanine when the system contained protein P10 derived from the parent strain, but there was little inhibition when P10 was derived from the resistant mutant. The other 30S ribosomal proteins did not significantly affect the inhibition by the antibiotics.

The stimulation of isoleucine incorporation with poly U in the presence of KM or SM was also dependent on the origin of P10 protein, but not on that of the other 30S ribosomal proteins. Cross-resistance to the two antibiotics was also observed in this experiment.

## Discussion

The alteration which led to KM and SM resistances was associated with 30S ribosomal subunits, not with 50S ribosomal subunits. Moreover, it was demonstrated that the alteration was in 23S core of 30S subunits, and the origin of SP30 did not affect the sensitivities to KM and SM. These results are in accord with previous studies[7,9,28]. The present studies further indicated that the determinant component for KM or SM sensitivity was protein P10 in the fractionated 30S ribosomal proteins.

The sensitivity to KM and that to SM are determined by the same ribosomal protein. In addition, the protein from either KM-resistant cells or SM-resistant cells showed resistance to both antibiotics in the present investigation. This suggests that the site of action of KM and that of SM are identical, or are overlapped mutually on the ribosomes.

The KM- and SM-resistant mutants employed showed one-way cross-resistance to the antibiotics. Almost the same pattern of resistance was demonstrated at the level of *in vitro* polypeptide-synthesizing system, using 70S ribosomes. However, the mutual cross-resistance of SM and KM was observed with dissociated and reconstituted 30S ribosomes. Although this discrepancy is inexplicable, it is possible that other ribosomal proteins as well as P10 or some other factors may participate in the resistance to the antibiotics.

Although P10 protein in the fractionated ribosomal proteins appeared to be a single

protein, it remains to be determined. The P 10 protein from parent strain and that from resistant mutant exhibited a different behavior on CMC column chromatography, suggesting a change in charge and therefore in the amino acid components.

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