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Analysis of Ribosomes from Viomycin-Sensitive and -Resistant Strains of *Mycobacterium smegmatis*

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Viomycin-resistant strains were isolated from *Mycobacterium smegmatis*. Ribosomes were isolated and tested for drug resistance in subcellular systems containing poly(U) as messenger ribonucleic acid. Resistance to viomycin in these strains was due to altered ribosomes. Further analysis showed that viomycin resistance of two mutants with low level resistance (20 μg/ml) was due to altered 30*S* ribosomal subunits. Another mutant that was highly resistant to viomycin (1 mg/ml), however, had altered 50*S* ribosomal subunits.

Although viomycin inhibits polypeptide synthesis in subcellular systems of *Escherichia coli* (8, 17), cultures of *E. coli* are rather resistant to this antibiotic (19). Because of this, attempts to isolate viomycin-resistant ribosomes from *E. coli* have not been reported (19).

Since mycobacteria are especially sensitive to viomycin (1), it was reasonable to expect that ribosomal resistant strains from mycobacteria might be obtained. This was found to be the case, and the findings are reported here including determination of the locus of viomycin resistance. The main conclusion is that viomycin action can probably be counteracted to some extent by changes in both 30*S* and 50*S* ribosomal subunits.

MATERIALS AND METHODS

Reagents and drug. Transfer ribonucleic acid (RNA) (*E. coli*) and poly(U) (molecular weight 10^5) were obtained from Miles Laboratories, Inc. Phosphoenolpyruvate monopotassium salt and phosphoenolpyruvate kinase were obtained from Sigma Chemical Co. ^{14}C-phenylalanine was obtained from Daiichi Pure Chemicals Co., Ltd. (Japan). Viomycin was a product of Pfizer Ltd. (Japan).

Strains and culture medium. *Mycobacterium smegmatis* ATCC 14468 and *E. coli* A 19 (ribonuclease I$^-$) were used. A strain of *M. smegmatis* 14468 was obtained from American Type Culture Collection, Rockville, Md., in 1966 and was maintained in our laboratory thereafter. *E. coli* A 19 (ribonuclease I$^-$) was obtained from R. F. Gesteland through K. Nakajima.

The culture medium for *M. smegmatis* contained the following ingredients, per liter of distilled water: broth, 10 g; polypeptone, 10 g; NaCl, 2 g; glycerol, 40 ml. The final *p*H was adjusted to 7.0 with 10% NaOH. Plates for cultivation of *M. smegmatis* contained the same ingredients supplemented with 1.75% agar. The culture medium for *E. coli* contained the following ingredients, per liter of distilled water: Na_2HPO_4, 5.8 g; KH_2PO_4, 3.0 g; NaCl, 5.0 g; NH_4Cl, 10.0 g; polypeptone, 10.0 g; glucose, 10.0 g. The final *p*H was adjusted to 7.0.

Isolation of viomycin-resistant strains. Resistant strains of *M. smegmatis* were obtained by serial transfers of the culture to the medium containing increasing amounts of viomycin. Cultivation was carried out at 37 C by shaking. The growing cultures were filtered through cellulose acetate filters (pore size, 3 μm), and then the filtrates were inoculated on glycerol agar plates containing appropriate amounts of viomycin. After incubation for 4 days at 37 C, viomycin-resistant strains grown on the plates were isolated. Among several viomycin-resistant strains, three strains (designated as A, E, and M) were selected and used in this experiment. Strain A was highly resistant to viomycin (1 mg/ml), and strains E and M had low level resistance (10 μg/ml to 20 μg/ml). Growth of the parent strain was inhibited by 0.6 to 1 μg of viomycin per ml.

Ribosomes and supernatant fluid. Organisms were cultured in rotary shaking flasks at 37 C. They were harvested at an optical density at 590 nm of 1.5 to 2.5. After three washes with a standard buffer [0.01 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.8; 0.06 M NH_4Cl; 0.006 M 2-mercaptoethanol] containing 10^{-2} M $MgCl_2$, the cells were suspended in twofold volumes of the same buffer and disrupted by sonic oscillation (Tomy model UR-150 P [Japan]). Cellular debris was removed by centrifugation at 20,000 × *g* for 30 min. Centrifugation of the re-

sulting crude extract was repeated two more times. Ribosomes and supernatant fluid were separated by centrifuging crude extracts at 104,500 × *g* for 120 min. The supernatant fraction was dialyzed against 600-fold volumes of standard buffer containing 10^{-2} M $MgCl_2$ for 24 hr. It was divided into small portions and stored at −70 C until use. Ribosomes were washed by centrifugation at 68,000 × *g* for 15 hr through buffer consisting of 0.05 M Tris (*p*H 7.6), 0.01 M $MgCl_2$, 0.1 M NH_4Cl, 0.001 M ethylenediaminetetraacetate, and 0.0005 M Cleland's reagent containing 34% sucrose as described by Capecchi (6). Washed ribosomes were suspended in standard buffer containing 10^{-2} M $MgCl_2$ and stored at −70 C until use. For isolation of subunits, ribosomes were dialyzed against standard buffer containing 10^{-4} M $MgCl_2$ for 18 hr at 4 C. Samples were loaded on sucrose gradients (10 to 30%) in the same buffer, and centrifugation was carried out at 21,000 rev/min for 12 hr in a Spinco SW25.2 rotor. Fractions were collected and monitored for absorption at 260 nm (A_{260}). The 50*S* and 30*S* fractions were pooled and centrifuged at 78,000 × *g* for 20 hr. The pellets were resuspended in standard buffer containing 10^{-2} M $MgCl_2$ and stored at −70 C until use.

Cell-free system. Assay of polyphenylalanine synthesis was carried out as described by Nirenberg (12) with a few minor modifications. The standard reaction mixture (0.1 ml) contained the following: 95 mM Tris, *p*H 7.8; 5.8 mM 2-mercaptoethanol; 7.5 mM phosphoenolpyruvate monopotassium salt; 0.9 mM adenosine triphosphate; 0.028 mM guanosine triphosphate; 0.1 mM ^{14}C-phenylalanine (specific activity, 100 μCi/5.5 μmoles); 0.186 mM each of nineteen other ^{12}C-amino acids; 48 mM NH_4Cl; 1.5 μg of phosphoenolpyruvate kinase; 20 μliters of supernatant fluid (A_{280}, 25); 120 to 180 μg of ribosomes; 20 μg of poly(U); 50 μg of *E. coli* transfer RNA; and 8 mM $MgCl_2$. After the reaction mixture was incubated at 37 C for 45 min, the reaction was stopped by adding 1 ml of 10% trichloroacetic acid, and the mixture was incubated for an additional 30 min at 95 C. The resulting acid-insoluble materials were collected on a membrane filter (Millipore; pore size, 0.45 μm). Radioactivity was counted by liquid scintillation spectrometer.

RESULTS

Effect of viomycin on ribosome function. A cell-free system containing ribosomes and supernatant fluid from wild-type, sensitive *M. smegmatis* was made and tested for viomycin sensitivity at various concentrations of antibiotic. As shown in Fig. 1, poly(U)-directed polyphenylalanine synthesis was almost completely inhibited at a concentration of 1 μg of viomycin per ml.

A similar system from resistant strain A was also tested, and viomycin sensitivity of the system was compared to that from the parent strain (Fig. 1). Slight stimulation but no inhibition of the resistant system was observed at a

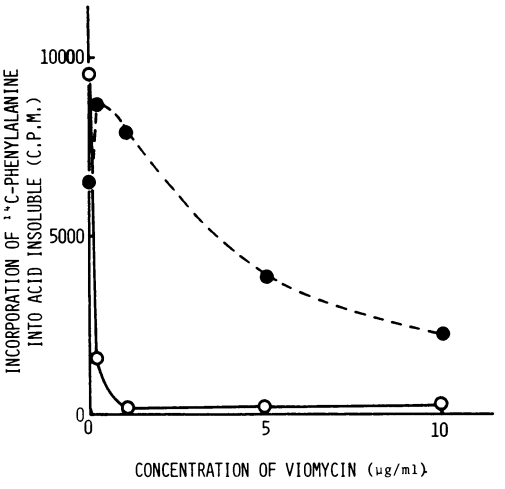

FIG. 1. *Poly(U)-directed polyphenylalanine synthesis in cell-free systems derived from viomycin-sensitive or -resistant strains in the presence of various concentrations of viomycin. Various amounts of viomycin were added to standard reaction mixtures, described in the text, containing ribosomes and supernatant fluid from either a sensitive* (○), *or resistant* (●) *strain (strain A) of M. smegmatis. Viomycin was added at the onset of incubation; assay procedures were as described in the text. The radioactivity in the absence of poly(U) (300 to 400 counts/min) was subtracted from each point.*

viomycin concentration of 1 μg/ml. With increasing drug concentration partial inhibition was seen (Fig. 1). Strain E, showing a different level of viomycin resistance, was compared to strain A in a similar experiment. Extracts of strain E showed a lower resistance to viomycin (Fig. 2), and this resistance paralleled that of cultures of this strain. Also, cultures of strain A are more resistant than cultures of strain E, and extracts from A were more resistant than those of E.

To determine whether viomycin resistance was due to an alteration in ribosomes or supernatant fraction, these two components were exchanged between parent and resistant strain A in the cell-free system. Each system was tested for viomycin sensitivity at a low concentration of antibiotic. The results (Table 1) show that viomycin resistance is determined by the source of the ribosomes, and thus the mutation must cause a ribosomal alteration. The system composed of sensitive ribosomes and resistant supernatant fluid was inhibited strongly by 1 μg of viomycin per ml, whereas that composed of ribosomes from strain A and supernatant fluid from the sensitive strain was resistant to viomycin.

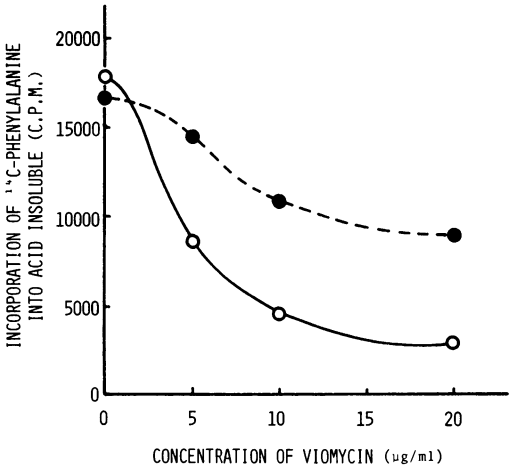

FIG. 2. *Poly(U)-directed polyphenylalanine synthesis in cell-free systems derived from two different viomycin-resistant strains. Various amounts of viomycin were added to standard reaction mixtures containing ribosomes and supernatant fluid from either strain A (●) or strain E (○) of M. smegmatis. Experimental procedures were identical with those in Fig. 1. The radioactivity in the absence of poly(U) (300 to 400 counts/min) was subtracted from each point.*

TABLE 1. *Effect of viomycin on phenylalanine incorporation in poly(U) system*[a]

Source of ribosomes	Source of supernatant	Viomycin[b] (μg/ml)	Incorporation[c] (Counts/min)
Sensitive	Resistant	0	7,382
Sensitive	Resistant	0.1	5,014
Sensitive	Resistant	1.0	490
Resistant	Sensitive	0	12,371
Resistant	Sensitive	0.1	13,349
Resistant	Sensitive	1.0	12,149

[a] Experimental procedures were described in the text.

[b] Antibiotic was added at the onset of incubation.

[c] The radioactivity (200 to 500 counts/min) in the absence of poly(U) was subtracted from radioactivity of each reaction.

The supernatant fraction from *E. coli* A 19 (ribonuclease I^-) was active for polyphenylalanine synthesis on ribosomes of *M. smegmatis* (T. Yamada, K. Kawaguchi, K. Masuda, K. Shoji, and M. Hori, Amer. Rev. Resp. Dis., *in press*), and this system was sensitive to viomycin. Since it was easy to prepare active supernatant fractions from *E. coli* A 19, the supernatant fluid fractions derived from *E. coli* were used for further analysis of altered ribosomes throughout succeeding experiments.

Characterization of low level resistant strains E and M. The 30*S* and 50*S* ribosomal subunits were isolated from parent and resistant strain E. Isolated subunits were exchanged between these strains to give reconstructed hybrid 70*S* ribosomes. Viomycin sensitivity of these hybrids was tested in a cell-free system. Since the optimal concentration of $MgCl_2$ for maximum polyphenylalanine synthesis on reconstructed or reconstituted ribosomes was 15 mM (Yamada et al., Amer. Rev. Resp. Dis., *in press*), the concentrations of magnesium ion were adjusted to give 15 mM in cell-free systems. As shown in Table 2, reconstructed ribosomes containing 30*S* subunits from strain E were resistant to inhibition of polyphenylalanine synthesis by viomycin. On the contrary, the hybrids composed of 50*S* subunits from the resistant strain and 30*S* subunits from the sensitive strain were sensitive, as were reconstituted parental 70*S* ribosomes. Similar experiments were carried out with strain M. The results are summarized in Table 3. We concluded from these experiments that resistance was due to altered 30*S* subunits in strains E and M.

Characterization of high level resistant strain A. Hybrid ribosomes were reconstructed from the parent sensitive strain and resistant strain A and tested for viomycin sen-

TABLE 2. *Analysis of ribosomal subunits from low level resistant strain E in poly(U) system*[a]

Constitution of hybrids[b]		Viomycin[c] (μg/ml)	Incorporation[d]	
50*S*	30*S*		Counts/min	%
Sensitive	Sensitive	0	4,150	100
Sensitive	Sensitive	0.5	693	14
Resistant	Resistant	0	2,364	100
Resistant	Resistant	0.5	2,010	85
Resistant	Sensitive	0	4,708	100
Resistant	Sensitive	0.5	1,505	32
Sensitive	Resistant	0	2,428	100
Sensitive	Resistant	0.5	2,058	85

[a] Experimental procedures were similar to those in Table 1 with the exception that magnesium chloride was used at 15 mM.

[b] Each reaction mixture contained 120 μg of 50*S* subunits and 60 μg of 30*S* subunits.

[c] Antibiotic was added at the onset of incubation.

[d] The radioactivity (200 to 250 counts/min) in the absence of poly(U) was subtracted from radioactivity of each reaction. Significant stimulation of incorporation by adding poly(U) was not observed on either 50*S* or 30*S* subunits alone.

TABLE 3. *Analysis of ribosomal subunits from low level resistant strain M in poly(U) system*[a]

Constitution of hybrids		Viomycin (μg/ml)	Incorporation	
50S	30S		Counts/min	%
Sensitive	Sensitive	0	3,394	100
Sensitive	Sensitive	1	377	11
Resistant	Resistant	0	4,779	100
Resistant	Resistant	1	2,814	59
Resistant	Sensitive	0	5,256	100
Resistant	Sensitive	1	673	13
Sensitive	Resistant	0	5,109	100
Sensitive	Resistant	1	2,055	40

[a] See footnotes to Table 2 for experimental details.

sitivity in cell-free systems. Poly(U)-directed polyphenylalanine synthesis on reconstituted sensitive 70S ribosomes was almost completely inhibited at a viomycin concentration of 1 μg/ml, and no inhibition was observed on reconstituted resistant ribosomes, as shown in the first and second columns of Table 4. On the other hand, a reconstructed hybrid composed of resistant 50S and sensitive 30S subunits was resistant to inhibition by viomycin, whereas the hybrid composed of sensitive 50S and resistant 30S was sensitive to the inhibition (third and fourth columns of Table 4). Thus, we concluded that 50S subunits from the resistant strain A were responsible for viomycin resistance, in contrast to strains E and M.

Exchange of subunits between strains A and M. To confirm the above conclusion, the 50S and 30S subunits from the two kinds of resistant strains A and M were exchanged to produce hybrids and were tested for viomycin sensitivity. One of the hybrids should give the parental sensitive phenotype, and the other should give a resistant phenotype. That this was the case is shown in Fig. 3. The hybrid ribosomes composed of 50S subunits from strain M and 30S subunits from strain A gave a parental, sensitive phenotype. In Fig. 3, the extent of inhibition by increasing concentrations of viomycin is shown. The hybrid composed of 50S subunits from strain A and 30S subunits from strain M were strongly resistant and did not show inhibition even at high concentrations of viomycin, compared with 70S ribosomes from strain A shown in Fig. 1 and 2.

TABLE 4. *Analysis of ribosomal subunits from high level resistant strain A in poly(U) system*[a]

Constitution of hybrids		Viomycin (μg/ml)	Incorporation	
50S	30S		Counts/min	%
Sensitive	Sensitive	0	3,120	100
Sensitive	Sensitive	1	481	15
Resistant	Resistant	0	3,086	100
Resistant	Resistant	1	3,320	107
Resistant	Sensitive	0	2,223	100
Resistant	Sensitive	1	3,426	153
Sensitive	Resistant	0	2,291	100
Sensitive	Resistant	1	440	19

[a] See footnotes to Table 2 for experimental details.

DISCUSSION

Viomycin-resistant strains of *M. smegmatis* were isolated by serial transfers of parental

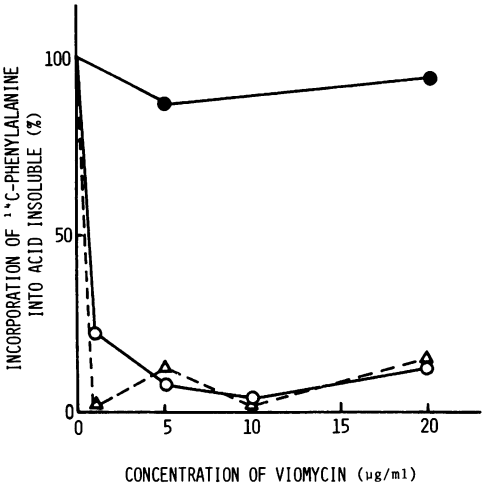

FIG. 3. *Effect of viomycin on polyphenylalanine synthesis on hybrid ribosomes composed of subunits derived from strain A and strain M. Symbols:* ●, *hybrid ribosomes composed of 50S subunits from strain A and 30S subunits from strain M;* △, *hybrid ribosomes composed of 50S subunits from strain M and 30S subunits from strain A;* ○, *reconstituted parental ribosomes. Incubation conditions were similar to those described in Fig. 1 with the following exceptions. Magnesium chloride was used at 15 mM. Each reaction mixture contained 120 μg of 50S subunits and 60 μg of 30S subunits. Results are expressed as percent of polyphenylalanine synthesis. Incorporation of 100% corresponds to that observed on respective ribosomes in the absence of viomycin. Significant stimulation of incorporation by adding poly(U) was not observed on either 50S or 30S subunits alone.*

cells to medium containing increasing concentrations of antibiotic. The isolation of mutants by a one-step selection was without success, except for the appearance of low level resistant strains (5 μg/ml). The reasons for these difficulties are unknown, but a possible explanation would be that viomycin might act at multiple sites of the protein synthesizing machinery. Another possibility would be that the specific translation component that is the site of viomycin action might require multiple mutations for phenotypic expression of resistance.

Studies of cell-free systems from resistant strains show that viomycin is an inhibitor of ribosome function (Table 1, Fig. 1 and 2). This conclusion was also supported by the failure to demonstrate inhibition of aminoacylation of transfer RNA by viomycin (*unpublished data*).

From the comparison between Fig. 1 and Fig. 2, it was seen that the extent of inhibition of polyphenylalanine synthesis by viomycin was variable in the system from strain A especially at high concentrations of the antibiotic. But no inhibition was observed below a viomycin concentration of 1 μg/ml throughout this work. On the other hand, partial inhibition (between 15 and 30%) of polyphenylalanine synthesis by viomycin at a concentration of 0.5 or 1 μg/ml was also reproducible in the system from strain E (Table 2).

Further analysis showed that viomycin resistance was due to either altered 50*S* or altered 30*S* subunits depending on the resistant strain. We observed altered 30*S* ribosomal subunits from two of the low level resistant strains and altered 50*S* subunits from one of the high level resistant strains. It is not clear, however, that high level resistance is always due to altered 50*S* subunits and low level resistance to altered 30*S* subunits. Low concentrations of viomycin inhibit, almost completely, the elongation of polyphenylalanine chains (probably translocation or peptidyl transferase), which is presumed to be a function of the 50*S* subunits.

From these observations, we suggest that the primary action of viomycin was on 50*S* function, with a secondary effect at higher drug concentrations on 30*S* function. Therefore, ribosomes containing altered 50*S* subunits would be completely resistant at low concentrations of drug and would become partially drug sensitive on increasing the drug concentration, reflecting partial inactivation of 30*S* function as shown in Fig. 1 and 2.

On the other hand, one could expect that ribosomes containing altered 30*S* and parental sensitive 50*S* subunits might be sensitive at a low viomycin concentration, since 50*S* subunits are presumed to be the primary target for viomycin. We also consider the possibility that the response of 50*S* subunits to viomycin could be modified by association with altered 30*S* subunits into 70*S* ribosomes.

Finally, by presenting evidence that hybrid ribosomes containing altered 50*S* subunits from strain A and altered 30*S* subunits from strain M were strongly resistant even at high concentrations of viomycin, it was concluded that viomycin action could probably be counteracted to some extent by changes in both 50*S* and 30*S* subunits.

Resistance to streptomycin and spectinomycin has been studied extensively. The mechanism of low level resistance to streptomycin and spectinomycin (20 μg/ml), which is mediated by an R factor, is enzymatic inactivation (3, 14, 18, 20). The high level resistance to streptomycin and spectinomycin (1 mg/ml) was isolated by single-step selection in *E. coli*. The localization of streptomycin and spectinomycin resistance on 30*S* subunits has been reported (7, 9, 10). Further analysis of altered 30*S* subunits has shown changes of specific proteins of 30*S* subunits (4, 5, 13, 15).

In mutants of *E. coli* resistant to erythromycin or lincomycin, a specific protein associated with 50*S* subunits seems to be altered (11, 16).

The chemical changes of ribosomal RNA and proteins of both 50*S* and 30*S* subunits have been reported in showdomycin-resistant *E. coli* (2). Drastic changes of chemical composition of ribosomes from showdomycin-resistant *E. coli* may be due to the inducing action of the nucleoside antibiotic (2). Until now, the observation that an antibiotic action can be affected by functional alteration of both 30*S* and 50*S* subunits has not been reported (19). In this work, alteration of either 50*S* or 30*S* subunits resulted in viomycin resistance to some extent, and a hybrid composed of altered 50*S* and altered 30*S* subunits gave strong resistance even at higher concentrations of viomycin. Chemical and functional characterization of the ribosomal protein of altered subunits will be required to establish the localization of resistance rigorously.

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