ON THE MODE OF ACTION OF MULTHIOMYCIN. II

EFFECTS OF MULTHIOMYCIN ON phe-tRNA BINDING TO RIBOSOMES AND ON OTHER STEPS IN PROTEIN SYNTHESIS

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Multhiomycin inhibited phe-tRNA binding to ribosomes at magnesium concentration greater than 10 mM. Binding of phe-oligonucleotide (RNase T_1 digest of phe-tRNA), to ribosomes and the reaction of puromycin with N-acetyl-phe-tRNA in the presence of GTP and S 105 supernatant fraction were not affected. The antibiotic inhibited phe-tRNA binding to the 30S ribosomal subunit. Dipeptide synthesis between phe-tRNA and N-acetyl-phe-tRNA which was prebound to ribosomes was strongly inhibited at 10 mM Mg++. The results suggest that multhiomycin inhibits phe-tRNA binding to amino-acyl site (A site) on the ribosomes.

It has been demonstrated that multhiomycin, a sulphur-containing antibiotic[1], inhibited protein synthesis in *Bacillus subtilis* cells and *Escherichia coli* lamelloplasts. In a cell-free system from *E. coli*, it inhibited polypeptide synthesis directed by native messenger and poly U. An inhibitory effect of multhiomycin on aminoacylation of tRNA or attachment of poly U to ribosomes was not observed[2].

Effects of the antibiotic on various steps in protein synthesis is described in this communication with the results suggesting that multhiomycin inhibits phe-tRNA binding to the aminoacyl site (the A site) on the ribosomes.

Materials and Methods

^{14}C-Phenylalanine (475 mC/mM) and ^{3}H-phenylalanine (1 C/mM) were purchased from Radiochemical Center.

Ribosomes, 105,000 × *g* supernatant fraction (S 105), tRNA***, [^{14}C]phe-tRNA and [^{3}H]-phe-tRNA were prepared as previously described[2]. [^{3}H]phe-tRNA was acetylated with acetic anhydride according to the procedure of HAENNI and CHAPEVILLE[3]. One mg of t-RNA was charged with 430 μμmoles (Fig. 1, Table 1), 224 μμmoles (Tables 3, 4) of ^{14}C-phenylalanine and with 63.6 μμmoles of ^{3}H-phenylalanine.

[^{14}C]Phe-oligonucleotide, a RNase T_1 digest of [^{14}C]phe-tRNA, was prepared by the

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** Abbreviations used : tRNA, transfer RNA; [^{14}C]phe-tRNA, tRNA charged with [^{14}C]phenyl-alanine, phe-oligonucleotide, phenylalanyl-oligonucleotide; N-acetyl-phe-tRNA, N-acetyl-phenyl-alanyl-tRNA; poly U, polyuridylic acid; N-acetyl-diphenylalanine, N-acetyl-phenylalanyl-phenyl-alanine; AA-tRNA, aminoacyl-tRNA; β-ME, β-mercaptoethanol.

method of HERBERT and SMITH[4]. The reaction mixture (4 ml) containing 4 mg [^{14}C]phe-tRNA (119,600 cpm), 8 μM EDTA, 40 μM potassium acetate (pH 5.2) and 400 units RNase T$_1$ (Sankyo Co.) was incubated for 30 minutes at 37°C. The digest was placed on a DEAE-cellulose column and stepwise elution was carried out with increasing concentrations of ammonium formate buffer[4]. [^{14}C]Phe–oligonucleotide eluted from the column was precipitated by the addition of 2.5 volume of ethanol.

30 S ribosomal subunits were obtained by dialyzing 70 S ribosomes against 10 mM Tris-HCl buffer (pH 7.4) containing 10^{-4}M magnesium acetate, followed by centrifugation through 5~20 % sucrose gradient in the same buffer in a Backman SW 25.1 rotor at 22,000 rpm for 16 hours. The subunits were concentrated by centrifugation at 170,000 × g for 4 hours in an RP 55 rotor (Hitachi Co., Ltd.).

Tetracycline and puromycin were purchased from Nihon Lederle Co., Ltd. and Nutritional Biochemicals respectively.

Multhiomycin was dissolved in dimethylformamide (DMF) at a concentration of 10 mg/ml. The antibiotic solution was freshly prepared before each experiment.
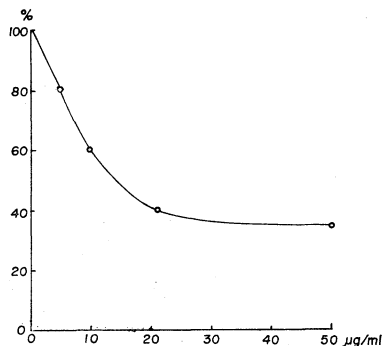
Results

Effect of Multhiomycin on the Binding of Phe–tRNA to Ribosomes

The binding of [^{14}C]phe–tRNA to ribosomes at 15 mM Mg^{++} concentration was studied with different concentrations of the antibiotic. As shown in Fig. 1, multhiomycin at concentrations of 20 μg/ml and 50 μg/ml inhibited the binding reaction to an extent of 60 % and 65 % respectively.

Table 1 illustrates the binding of phe–tRNA to ribosomes as a function of the Mg^{++} concentration. Phe–tRNA binding was slightly inhibited by multhiomycin at the lower Mg^{++} concentration, but as the Mg^{++} concentration increased, the inhibitory effect of multhiomycin became greater. At 15 mM Mg^{++} concentration, tetracycline inhibited the binding reaction to an extent of 71 % and little enhancement of the inhibitory effect of this antibiotic was observed by the addition of multhiomycin to the reaction mixture.

Fig. 1. Binding of phe–tRNA to ribosomes at 15 mM Mg^{++} as a function of multhiomycin concentration.

The reaction mixture (0.2 ml) contained 1 μl DMF, 10 μmoles Tris–HCl (pH 7.4), 10 μmoles KCl, 3 μmoles magnesium acetate, 1.2 μmoles β-ME, 10 μg poly U, 0.2 mg ribosomes, 58 μg [^{14}C]phe–tRNA (18,000 cpm) and the indicated amounts of multhiomycin. Incubations were performed at 30°C for 12 minutes and diluted by addition of 3 ml of cold 0.05 M Tris–HCl buffer containing 0.05 M KCl and 0.015 M magnesium acetate. [^{14}C]Phe–tRNA bound to ribosomes was determined by the Millipore filter technique[25].

Table 1. Binding of [^{14}C]phe–tRNA to ribosomes at different Mg^{++} concentration.

The binding reaction was carried out as described in Fig. 1 except that the Mg^{++} concentration was varied. Multhiomycin (50 μg/ml) and tetracycline (5 × 10^{-4}M) were added where indicated.

Addition	Mg^{++}		
	5 mM	10 mM	15 mM
Control	190[1] (100)[2]	713(100)	1362(100)
Multhiomycin	176 (92)	336 (47)	446 (33)
Tetracycline	——	——	396 (29)
Multhiomycin + Tetracycline	——	——	325 (23)

1) [^{14}C]phe–tRNA bound to ribosomes (cpm)
2) Data in parenthesis represent percent of control at each Mg^{++} concentration.

Effect of Multhiomycin on the Binding of [^{14}C]Phe–oligonucleotide
to Ribosomes

A ribonuclease T_1 digest of phe–tRNA, phe–oligonucleotide, can bind to 70S ribo-somes; this reaction represents the binding of the aminoacyl end of aminoacyl–tRNA to ribosomes[5].

Table 2 shows that multhiomycin did not inhibit the binding of this fragment to ribosomes even at a concentration of 50 μg/ml. Chloramphenicol, as already reported, inhibited this reaction[5,6] and blasticidin S, an inhibitor of peptidyl transfer[7], also inhibited the binding completely at a concentration of 2.3×10^{-4}M.

Effect of Multhiomycin on [^{14}C]phe–tRNA Binding to 30S Ribosomal Subunit

One molecule of aminoacyl–tRNA binds to the 30S ribosomal subunit–mRNA complex[8] under conditions where the codon–anti-codon interaction is involved.

Multhiomycin significantly af-fected the binding reaction as shown in Table 3.

Effect of Multhiomycin on N–Acetyl–phe–puromycin Formation

The peptide bond forming re-action between peptidyl–tRNA on peptidyl site (the P site) and AA–tRNA on aminoacyl site (the A site) is catalyzed by peptidyltransferase which is an integral part of the ribosome[9~13]. The reaction of puromycin with N–acetyl–phe–tRNA bound to the P site (puro-mycin reaction) occurs readily to form N–acetyl–phe–puromycin[14,15]. This reaction is stimulated by GTP and G factor which translocates N–acetyl–phe–tRNA from the A site to the P site. As shown in Table 4, multhiomycin did not inhibit

Table 2. Binding of [^{14}C]phe–oligonucleotide to ribosomes.

The reaction mixture (0.1 ml) contained 0.5 μl DMF, 5 μ moles Tris HCl (pH 7.2), 2 μ moles magnesium acetate, 40 μ moles KCl, 200 μg ribosomes, [^{14}C]phe–oligonucleotide (4,390 cpm) and was incubated at 24℃ for 10 minutes. Binding of [^{14}C]phe–oligonucleotide to ribosomes was as-sayed by the Millipore filter method[25].

	[^{14}C]Phe–oligonu-cleotide bound to ribosomes (cpm)	Percent of complete
Complete	207	100
+ poly U	213	103
+ Multhiomycin 50 μg/ml	203	98
+ Chloramphenicol 10^{-4}M	19	9
+ Blasticidin S 2.3×10^{-5}M	10	5

Table 3. Effect of multhiomycin on the binding of [^{14}C]phe–tRNA to 30S ribosomal subunits.

The raction mixture (0.2 ml) containing 10 μmoles Tris-HCl (pH 7.4), 10 μmoles KCl, 3 μmoles magnesium acetate, 1.2 μmoles β-ME, 10 μg poly U, 2 A_{260} units 30S ribosomal subunit, 33 μg [^{14}C]phe–tRNA (5,350 cpm) was incubated for 7 minutes at 37℃. [^{14}C]phe–tRNA bound to 30S ribosomal subunits was determined as described in the legend to Fig. 1.

	[^{14}C]Phe–tRNA bound (cpm)	Percent of control
Control	655	100
−poly U	51	8
Multhiomycin 50 μg/ml	242	37

Table 4. Reaction of puromycin with N–acetyl [^{3}H]phe–tRNA in the presence of antibiotics.

The complete reaction mixture containing 12.5 μmoles Tris-HCl (pH 7.4), 2.5 μmoles magnesium acetate, 12.5 μmoles KCl, 1.5 μmoles β-ME, 10 μg poly U, 0.38 mg ribosomes, 25 mμ moles GTP, 45 μg N–acetyl[^{3}H]phe–tRNA (3,700 cpm) in a total volume of 0.21 ml, was incubated for 6 minutes at 30℃. Each reaction mixture was further incubated with 0.04 ml of 2.5 % DMF solution containing 100 μg puromycin and other antibiotics as indicated. Reactions were stopped by addition of 1 ml of 0.1 M sodium acetate (pH 5.5) and 1.5 ml of ethyl acetate[27]. One milliliter of the ethyl acetate layer wes placed on a planchet, evaporated, and the radioactivity was counted with a gas-flow counter.

Additions or deletions	N–Acetylphepuromycin formed (cpm)
Complete	1,032
−Puromycin	221
−GTP, S 105	808
+Fusidic acid 10^{-5} M	846
+Multhiomycin 50 μg/ml	1,140
−Puromycin +Multhiomycin	290

N–acetyl–phe–puromycin formation in the presence of GTP and the S105 supernatant fraction indicating that the antibiotic inhibits neither peptide bond formation nor translocation.

Fusidic acid, which is an inhibitor of translocation, inhibited the puromycin reaction as stimulated by GTP and the S105 supernatant as already reported by TANAKA et al.[16]

Effect of Multhiomycin on N–Acetyl–diphenylalanine Formation

According to PESTKA[17], diphenylalanine and a small amount of oligophenylalanine are synthesized when ribosomes, poly U and phe–tRNA are incubated. Under our conditions, ribosomes were preincubated at 13 mM Mg^{++} with N–acetyl–[^{3}H]phe–tRNA in the presence of poly U for 30 minutes at 30°C. The reaction mixtures were exposed to antibiotics for 6 minutes and then [^{14}C]phe–tRNA was added to each sample. The final Mg^{++} concentration was 10 mM. In this experiment, only [^{14}C]phe–tRNA bound to the A site during the third stage of incubation may react with N–acetyl–phe–tRNA bound to the P site in the first incubation to form N–acetyl–[^{3}H] phe[^{14}C] phenylalanine. This product can be extracted with ethyl acetate together with N–acetyl[^{3}H]–phenylalanine after deacylation of tRNA bound to ribosomes. Thus, [^{14}C]phenylalanine extracted reflects [^{14}C]phe–tRNA binding to the A site.

As can be seen from Table 5, the release of ethyl acetate–extractable radioactivity was 85 % inhibited by 50 μg/ml of multhiomycin.

Table 5. Effect of antibiotics on N–acetyl [^{3}H]phe[^{14}C]phe formation.

The reaction mixture (0.15 ml) containing 10 μmoles Tris–HCl (pH 7.4), 10 μmoles KCl, 2 μmoles magnesium acetate, 1.2 μmoles β–ME, 10 μg poly U and 90 μg acetyl[^{3}H]phe–tRNA (7,400 cpm) was incubated at 30°C for 50 minutes and then 0.04 ml of antibiotic solution in 2.5 % DMF was added to each sample. After incubation at 30°C for 6 minutes, 0.01 ml of [^{14}C]phe–tRNA (47 μg, 7,400 cpm) was added and samples were further incubated at 30°C for 10 minutes. N–Acetyl[^{3}H]phe–tRNA and [^{14}C]phe–tRNA bound to ribosomes were adsorbed to Millipore filters, which were soaked in 3 ml of 0.3 N KOH at 37°C for 30 minutes. To each test tube, 0.1 ml of 12 N HCl was added and Millipore filters were removed. 1.5 ml of ethyl acetate was added and 1 ml of the solvent layer was transfered to a scintillation vial. Radioactivity was counted in a scintillation counter following the addition of 10 ml of toluene–PPO–POPOP solution[26].

	Radioactivity extracted with ethyl acetate (cpm)		Percent of control of ^{14}C counts
	^{3}H	^{14}C	
Contrnl	1, 893	87	100
– poly U	104	8	9
Multhiomycin 50 μg/ml	2, 018	15	17
Blasticidin S 2.3×10^{-5}M	2, 431	33	38

Discussion

In a previous report[2], it was reported that multhiomycin inhibited protein synthesis both in vivo and in vitro and that the primary site of action might be located in some step following the formation of the messenger–ribosome complex. The results presented in this communication supports the idea that multhiomycin inhibits the binding of amino-acyl–tRNA to the aminoacyl site (the A site) on the ribosomes.

It has been proposed that there are two binding sites for AA–tRNA on the 70S ribosomes, one is the aminoacyl site (the A site) and the other the peptidyl site (the P site). According to IGARASHI et al.[18], AA–tRNA preferentially binds to the P site at lower Mg^{++} concentration and to the both sites at higher Mg^{++} concentration. This phenomenon was used to study the effect of multhiomycin on phe–tRNA binding to ribosomes. The inhibitory effect of multhiomycin on the binding reaction was weak at 5 mM Mg^{++}, but increased as the Mg^{++} concentration was increased. At 15 mM Mg^{++} concentration, the antibiotic inhibited the reaction by 65 % at a concentration of 50 μg/ml. Inhibition

of the reaction by tetracycline, an inhibitor of AA–tRNA binding to the A site[19,20], was enhanced only slightly by the addition of multhiomycin (Table 1). If multhiomycin inhibits the binding of phe–tRNA to the both sites, inhibition by a mixture of the two antibiotics should exceed 90 %. These results suggest that multhiomycin inhibits phe–tRNA binding to the A site on the ribosomes.

Peptide bond formation on the ribosomes was assayed by N-acetyl-phe-puromycin formation. This reaction was not inhibited by multhiomycin in the presense of GTP and S 105, indicating that the antibiotic affects neither peptidyl transfer nor translocation of AA–tRNA from the A site to the P site.

It is thought that at least three regions in tRNA are involved in binding to ribosomes, these include anticodon, the aminoacyl end and the TψGC region[22].

Phe–oligonucleotide (CACCA–phe), the aminoacyl end of phe–tRNA, binds to ribosomes even in the presence of multhiomycin, suggesting that the antibiotic does not affect this interaction. Blasticidin S completely inhibited this binding reaction at 2.3×10^{-4}M. The nucleoside antibiotic gougerotin is also inhibitory as reported by PESTKA[6]. Recently KINOSHITA et al. indicated that blasticidin S binds reversibly to the 50S ribosomal subunit and this binding was prevented by gougerotin[21]. These results suggest that blasticidin S affects the 50S ribosomal subunit, resulting in inhibition of the binding of the aminoacyl end of tRNA.

The binding of phe–tRNA to the 30S ribosomal subunit which involves the codon-anticodon interaction was inhibited by multhiomycin as shown in Table 3. Since the 30S ribosomal subunit presumably has one AA–tRNA binding site which is considered to be the A site[8], this result is consistent with the speculation that the antibiotic inhibits AA–tRNA binding to the A site.

This was further substantiated by the fact that the formation of N-acetyl-[³H]phe-[¹⁴C]-phe from prebound N-acetyl-[³H]phe-tRNA and [¹⁴C]phe-tRNA was inhibited 85 % by the presence of multhiomycin. Since only [¹⁴C]phe-tRNA bound to the A site can react with N-acetyl-[³H]phe-tRNA on the P site, a reduction in the formation of N-acetyl-[³H]phe[¹⁴C]phe reflects the inhibition of phe–tRNA binding to the A site.

The data presented in this manuscript suggests that the mechanism of action of multhiomycin is closely related to that of tetracycline in that the both antibiotics inhibit phe–tRNA binding to the A site. Recent observations indicate that multhiomycin also inhibits T factor and GTP dependent binding (enzymatic binding) of phe–tRNA to ribosomes[23]. Since this enzymatic binding takes place on the A site[24], the inhibitory effect of multhiomycin on this reaction supports the notion that the antibiotic inhibits the binding of phe–tRNA to the A site on the ribosomes.

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