THE EFFECT OF VIOMYCIN ON ELONGATION STEPS IN CELL-FREE POLYPEPTIDE SYNTHESIS

TAKESHI YAMADA

Research Institute for Microbial Diseases,
Osaka University,
3-1 Yamada-oka, Suita-city, Osaka 565, Japan

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Viomycin (VM), differs from most antibiotics in being more active against mycobacteria than other bacterial species in culture[1,2]. The fact that VM inhibits protein synthesis was first shown in intact *Mycobacterium avium*[3] and it is a ribosomal inhibitor since VM resistance of certain mutants of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* can be attributed to an alteration of ribosomes[4~7].

The characteristics of VM binding to ribosomes have been studied. The drug reduced the amounts of [^{3}H]dihydrostreptomycin bound to ribosomes of *M. smegmatis* and *Escherichia coli* suggesting an interaction between the binding sites of these antibiotics[8]. [^{14}C]Tuberactinomycin O (TMO), VM derivative, bound to ribosomal RNA but not to ribosomal protein and blocked the *in vitro* assembly of *E. coli* 50S subunits[9]. [^{14}C]TMO bound to both *E. coli* 30S and 50S subunits[9,10]. The strong binding of [^{14}C]TMO to *M. smegmatis* 70S ribosomes and its weak affinity to either isolated subunits from *M. smegmatis* or 70S ribosomes from *E. coli* or VM-resistant *M. smegmatis* suggested the inhibitory effects of the drug are due to the association of VM-susceptible mycobacterial subunits resulting in potent inhibition of polypeptide synthesis in *Mycobacterium*[11].

Therefore, the drug probably influences functions in which both ribosomal subunits are involved. Such functions are 1) dissociation and association, 2) peptidyl tRNA binding to the P site and the A site, and 3) translocation. These possibilities were examined with the conclusion that VM inhibits dissociation of ribosomes. This effect of the drug was not seen with VM-resistant *M. smegmatis* ribosomes[12].

Since higher concentrations of VM inhibited poly(U)-directed polyphenylalanine synthesis in a *E. coli* cell-free system[13], detailed studies of the effect of VM on elongation steps were performed in this system.

No induction of misreading by VM has been reported[13,14], while the induction of the misreading at high VM concentrations was reported[15,16].

This antibiotic was reported to prevent both the binding of fMet-tRNA to the ribosomes and the translocation reaction in elongation processes in polypeptide synthesis[17], and has been confirmed by other groups[18,19].

I examine the effect of VM on elongation in *E. coli* cell-free translation system; I conclude that VM influence not only the translocation but also binding to the A site.

E. coli strain A19 was used in this study. Poly(U) and tRNA^{Phe} (*E. coli*) were obtained from Boehringer Mannheim, [^{14}C]Phe (504 mCi/mmol) from Amersham Co., Ltd., VM from Pfizer Taito Co., Ltd. Phe-tRNA was prepared and *N*-acetyled as described[20,21]. Nitrocellulose filters were from Sartorius, Gottingen (FRG). Elongation factor G (EF-G) was prepared according to ref 20. The assay system for elongation was based on the method of WATANABE[22] as modified by WURMBACH and NIERHAUS[16]. Briefly, the procedure consisted of three incubation steps. (i) Binding of tRNA^{Phe} and poly(U) to ribosomes: 225 μl of a reaction mixture containing 170 μg of poly(U), 180 pmol of 70S ribosomes, 300 pmol of tRNA^{Phe}, 50 mM Tris-HCl at pH 7.8, 10 mM MgOAc, 160 mM NH_2Cl, and 5 mM 2-mercaptoethanol was incubated for 10 minutes at 37°C. Under these conditions, the P site was precharged with tRNA^{Phe} and the A site was available for Ac-[^{14}C]Phe-tRNA^{Phe} binding. The same reaction mixture without tRNA^{Phe} was incubated at 37°C for 10 minutes to free the P site for Ac-[^{14}C]Phe-tRNA^{Phe} binding. Under such conditions, the puromycin reaction was positive only after incubation with EF-G. As a control, the same reaction mixture without tRNA^{Phe} and ribosomes was made. (ii) The binding of Ac-[^{14}C]Phe-tRNA^{Phe} to the P site and the A site. The volume of reaction mixture was increased to 450 μl upon addition of 72 pmol of Ac-[^{14}C]Phe-tRNA^{Phe}. The buffer contained 50 mM Tris-HCl at pH 7.8, 15 mM MgOAc, 160 mM NH_4Cl, and 5 mM 2-mercaptoethanol. After incubation for 30 minutes at 37°C, two 50-μl aliquots were removed and each was diluted with 1 ml of binding buffer (50 mM Tris-HCl, pH 7.5 - 15 mM MgOAc - 160

Table 1. The effect of viomycin on elongation steps of polypeptide synthesis.

Drug (μM)	Binding		Puromycin reaction				Efficiency of the translocation reaction[a]
	P site condition	A site condition	P site condition		A site condition		
			−EF-G	+EF-G	−EF-G	+EF-G	
0	6,198	3,184	4,922	5,310	84	1,923	
7	5,837	1,111	3,971	5,037	0	599	0.93
17	5,447	1,083	3,620	4,188	0	525	0.91
32	5,458	1,134	3,619	4,171	26	370	0.57
64	6,104	1,094	3,490	4,196	19	363	0.66

[a] The efficiency of the translocation reaction was calculated by the formula $(\Delta Apm/Ab \times P_b/Ppm)_D/(\Delta Apm/Ab \times P_b/Ppm)_{-D}$. ΔApm: The difference value of the puromycin reaction in the absence and the presence of EF-G, at the A site; P_b: the binding value at the P site condition; Ab: the binding value at the A site condition; Ppm: puromycin reaction at the P site condition in the presence of EF-G; $_D$: the value in the presence of the drug; $_{-D}$: the value in the absence of the drug.

mM NH_4Cl - 5 mM 2-mercaptoethanol). The solutions were filtered through nitrocellulose, washed 2 times with binding buffer and counted by liquid scintillation. (iii) To the remaining 350-μl reaction mixture, 70 μl of binding buffer containing 0.7 mM GTP, 7 mM phosphoenolpyruvate and 5 μg of pyruvate kinase were added. Six aliquots of 60 μl each were removed. Two reaction mixtures received 5 μl of buffer containing 10 mM Tris-HCl at pH 7.5, 10 mM MgOAc, 100 mM KCl, 10 mM 2-mercaptoethanol and 20% glycerol and 2 μg of EF-G; the other reaction mixtures received buffer without EF-G. The reaction mixtures were incubated at 37°C for 10 minutes to allow translocation to take place. Five μl of 10 mM puromycin in binding buffer was added to the reaction mixtures with and without EF-G and left on ice at least 30 minutes. The reaction was stopped by the addition of 65 μl of 0.3 M NaOAc at pH 5.5 saturated with $MgSO_4$ followed by 1 ml of EtOAc. After agitation with a vortex mixer for 1 minute, the mixtures were left on ice for 15 minutes and 0.7 ml of the EtOAc phase was removed and counted by liquid scintillation. The mean values of duplicates were calculated and corrected with control experiments.

Binding of Ac-[^{14}C]Phe-tRNA^{Phe} at the P site and the A site in the absence and the presence of 7 μM VM are presented in Table 1. The binding at the P site was not influenced significantly, whereas binding at the A site condition was reduced. In the P site reaction, 86% of the bound Ac-[^{14}C]Phe-tRNA^{Phe} reacted with puromycin indicating the binding to the P site was near quantitative. At the A site puromycin reaction was seen only after incubation with EF-G to allow translocation, indicating the binding at the A site. Therefore, it can be concluded that VM affects the binding affinity of Ac-[^{14}C]-Phe-tRNA^{Phe} to the A site but not to the P site of the ribosome.

The peptidyl transferase reaction was examined by measuring the puromycin reaction at the P site. The maximum activity, 5,310 counts, was obtained, when EF-G was added. As indicated above the efficiency of the peptidyl transferase reaction was 86%. The puromycin reaction in the presence of the drug was only slightly affected. The translocation reaction can be examined by the difference in the puromycin reaction in the presence and absence of EF-G. The results obtained in the presence of 7 μM VM are shown in Table 1.

The effects of varying VM concentration on binding to the P site and the A site, the puromycin reaction and translocation, is presented in Table 1. Binding at the P site and the peptidyl transferase reaction were not affected significantly by VM, whereas the binding to the A site and the translocation reaction was inhibited by increasing concentration of the drug. Inhibition of A site binding was detected at lower VM concentrations.

Inhibition of Ac-[^{14}C]Phe-tRNA^{Phe} binding to the ribosomal A site and inhibition of translocation are demonstrated. The inhibition of Ac-[^{14}C]Phe-tRNA^{Phe} binding is not surprising[16,19]. I emphasize that the extent of the inhibition varies between independent experiments. Thus, binding to the A site was only 25% of control in the presence of 40 μM VM in one experiment

whereas the same authors also reported 85% binding in the presence of the same concentration of VM in other experiments[16,19]. Other workers have reported no inhibition of Ac-[^{14}C]-Phe-tRNA^{Phe} binding to ribosomes[15,17]. However, in the latter, puromycin reaction was not used to establish the specificity of A site binding.

Although it is difficult to compare separate experiments a possible variable might be differences in concentration of NH_4Cl, which can influence the binding affinity of Ac-[^{14}C]Phe-tRNA^{Phe} to the A site[22]. Thus, inhibition by siomycin and tetracycline was observed at higher concentrations of NH_4Cl whereas no inhibition by siomycin was detected in the absence of NH_4Cl. The antibiotics did not influence the A site binding when added after incubating ribosomes and tRNA with Ac-[^{14}C]Phe-tRNA^{Phe}. In other reports[16,19] and in this paper, 160 mM NH_4Cl was used, while the other authors used 60 mM NH_4Cl[17]. Differences of ionic strength may possibly influence the A site binding in the presence of VM. Clarification of this point remains necessary.

I conclude that VM distorts only the translocation or both the translocation and the A site binding to cause inhibition depending on the environmental conditions.

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