SELECTIVE INHIBITION OF INITIATION OF GLOBIN SYNTHESIS BY PHENOMYCIN

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Summary

It has been demonstrated in a rabbit reticulocyte system that phenomycin selectively blocks the initiation of globin synthesis. The N-terminal incorporation of valine or methionine is more markedly inhibited by the antibiotic than the total incorporation. The 80S initiation complex is accumulated in the presence of phenomycin. The initial dipeptide formation (Met-Val) is significantly affected by the antibiotic.

Phenomycin is a basic polypeptide antibiotic (M.W. ca. 10,000), produced by Streptomyces fervens var. phenomyceticus (1). It exhibits a significant tumor-inhibitory activity against Ehrlich carcinoma, sarcoma 180, and adenocarcinoma 755 in mice; but no antimicrobial activity (2). The mechanism of action of phenomycin has been investigated with rat liver, Ehrlich carcinoma, and rabbit reticulocytes (3). The primary site of action is localized in the mammalian protein-synthesizing system. But it shows much less activity against bacterial protein synthesis. Therefore the selective toxicity of phenomycin is opposite to that of chloramphenicol. Phenomycin inhibits globin synthesis more significantly than polyphenylalanine synthesis in cell-free systems obtained from rabbit reticulocytes.

The mechanism of action of phenomycin has been further studied in a rabbit reticulocyte system. The results are presented in this publication. It has been observed that the antibiotic selectively inhibits the initiation of globin synthesis.

Materials and Methods

The rabbit reticulocytes and ribosomes with endogenous messengers were prepared by the method of Allen and Schweet (4); and the KCl-washed ribosomes by that of Miller and Schweet (5). The ribosomal wash with 0.5 M KCl was used as the crude initiation factor (5), and further purified by gel filtration (6). The preparation of elongation factors followed the procedure of McKeehan and Hardesty (7). The tRNA was extracted from rabbit liver by the method of McKeehan and Hardesty (7), and further purified by benzoylated DEAE cellulose chromatography (8). Bulk tRNA or $tRNA_f^{Met}$ of rabbit liver was acylated with methionine by E. coli aminoacyl-tRNA synthetase (9). The analysis of N-terminal methionine or valine of globin peptide was carried out by the PTC method (10). The reaction of Met-Val formation was performed by the modified procedure of Crystal et al. (11). Phenomycin was kindly given by Dr. Hamao Umezawa, Institute of Microbial Chemistry, Tokyo.

Table 1. Inhibition by phenomycin of valine incorporation into the N-terminus of globin in a cell-free system obtained from rabbit reticulocytes.

Inhibitor		[^{14}C]Val incorporated (pmoles/tube)		N-terminus / Total (%)	Inhibition (%)
		Total	N-terminus		
None		34.3	1.47	4.3	
Phenomycin	10^{-7} M	5.1	0.04	0.9	79
	10^{-8}	17.4	0.44	2.5	42
NaF	10^{-2} M	23.2	0.59	2.5	42

The reaction mixture contained: reticulocyte lysate 18 mg protein, ATP 2 mM, creatine phosphate 3 mM, creatine kinase 50 µg, GTP 0.05 mM, [^{14}C]valine 0.2 µCi, KCl 48 mM, $MgCl_2$ 4 mM, 2-mercaptoethanol 5 mM, Tris-HCl 8 mM, pH 7.5, in a total volume of 0.5 ml. The incubation was carried out at 37°C for 20 minutes.
The N-terminal valine analysis was performed by the PTC method. PTH-valine was identified by paperchromatography (ethyl acetate: n-hexane=1:1).

Results

The valine incorporation into the N-terminus of globin was more significantly affected by phenomycin than the total incorporation. In the absence of the antibiotic, 4.3 % of valine incorporated was found at the amino end of globin. In the presence of 10^{-7} M phenomycin, only 0.9 % of valine incorporated was observed at the N-terminus (Table 1).

The uptake of methionine into globin was studied in a globin-synthesizing system without tryptophan. In the absence of tryptophan, the short globin chain of 13 or 14 amino acid residues is formed, and the initiator Met-$tRNA_f$ is not removed from the N-terminus (12). Phenomycin was found to block the incorporation of methionine into the N-terminus more significantly than the total incorporation. At the concentration of 10^{-8} M of phenomycin 33% inhibition was observed (Table 2).

Table 2. The effect of phenomycin on methionine incorporation into the N-terminus of nascent globin peptide in a cell-free system.

Inhibitor		[^{3}H]Met incorporated (pmoles/tube)		N-terminus / Total (%)	Inhibition (%)
		Total	N-terminus		
None		8.49	0.87	10.2	
Phenomycin	10^{-8}M	7.29	0.52	6.80	33
	10^{-9}	8.06	0.71	8.75	14
Blasticidin S	10^{-5}M	2.25	0.33	12.6	-
NaF	10^{-2}M	6.32	0.40	6.3	38

The reaction mixture contained: Tris-HCl, pH 7.5, 10 mM, $MgCl_2$ 2.5 mM, dithiothreitol 0.5 mM, KCl 100 mM, ATP 1 mM, PEP 5 mM, pyruvate kinase 6 µg, GTP 0.2 mM, 17 amino acids except methionine and tryptophan 0.05 mM, rabbit liver tRNA 30 µg, reticulocyte ribosomes with endogenous messenger 12 A_{260} units, KCl-wash of ribosomes 60 µg protein, the enzymes of 0.4 - 0.7 $(NH_4)_2SO_4$ saturation fraction of S105 450 µg protein, and [^{3}H]methionine, in a total volume of 0.3 ml. The incubation was performed at 37°C for 20 minutes. The hot TCA-insoluble fraction was allowed to prepare PTH-protein and PTH-[^{3}H]methionine was extracted with ethyl acetate from the acid hydrolysate of PTH-protein.

Table 3. The effect of phenomycin on the formation of initial dipeptide Met-Val in a globin synthesis of rabbit reticulocytes.

Inhibitor	[^{35}S]Met (mpmoles)	[^{35}S]Met-Val (mpmoles)	Met-Val / Met + Met-Val	% Inhibition
(Exp. 1) None	12.74	2.34	0.185	
Phenomycin 10^{-6} M	11.20	1.40	0.125	33
10^{-7}	10.90	1.41	0.131	31
(Exp. 2) None	27.20	4.99	0.150	
Phenomycin 10^{-7} M	24.50	2.69	0.105	30
10^{-8}	27.63	4.15	0.130	13
(Exp. 3) None	19.90	2.82	0.142	
Phenomycin 10^{-7} M	20.30	1.65	0.082	42
ATA 2×10^{-4}	4.65	0.67	0.147	-
NaF 10^{-2}	5.33	0.74	0.139	2

The reaction mixture contained, in 0.4 ml: Tris-HCl, pH 7.5, 20 mM, KCl 100 mM, $MgCl_2$ 5 mM, dithiothreitol 1 mM, GTP 2 mM, ATP 1 mM, PEP 5 mM, pyruvate kinase 2 µg, $tRNA_f^{met}$ and $tRNA^{val}$ fraction 275 µg, [^{35}S]methionine 4 µCi, cold valine 0.05 mM, 0.5 M KCl-treated ribosomes 100 A_{260} units, EF T1 200 µg and aminoacyl-tRNA synthetase 40 µg. The incubation was carried out at 37°C for 20 min. and the reaction was terminated by the addition of 3 ml of chilled TMK buffer (Tris-HCl, pH 7.5, 20 mM, KCl 100 mM and $MgCl_2$ 5 mM) in an ice-cold bath. The product was collected on nitrocellulose filter, and washed three times with the same buffer. The filter was placed in 5 ml of 0.1 N $(NH_4)_2CO_3$, incubated at 37° C for 15 min. to release the oligopeptide products, lyophilized, and dissolved in a drop of H_2O with authentic methionine and methionylvaline as markers. The solution was spotted on paper and subjected to electrophoresis at pH 3.5 (pyridine:acetic acid: H_2O=1:10:89) at 900 V for 7 hours. The paper was cut out, and the radioactivity was determined by a scintillation counter. The sharp peaks of [^{35}S]counts were obtained corresponding with methionine and methionylvaline spots, which were detected by spraying with 0.05 N iodine in 50 % ethanol containing 1.5 % sodium azide.

In the simultaneous experiments, sodium fluoride, an inhibitor of initiation of protein synthesis (13), was observed to show the same tendency of inhibition as phenomycin; but blasticidin S, an inhibitor of peptide chain elongation (14), did not selectively affect the N-terminal incorporation.

The formation of initial dipeptide Met-Val in globin synthesis was significantly blocked by phenomycin: 30 or 42 % inhibition was observed at the concentration of 10^{-7} M (Table 3). The grade of

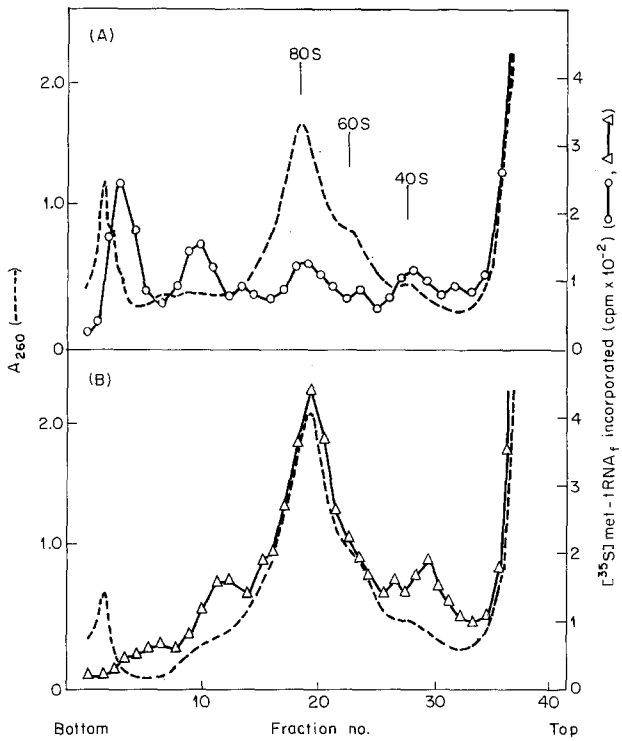

Fig. 1. The effect of phenomycin on the incorporation of [^{35}S]Met-tRNA$_f$ to the reticulocyte ribosomes.

The reaction mixture contained, 0.2 ml: Tris-HCl, pH 7.5, 20 mM, $MgCl_2$ 3 mM, KCl 75 mM, dithiothreitol 1 mM, 0.4-0.7 saturation fraction of S105 550 µg, ribosomes with endogenous messenger 10 A_{260} units, ATP 1 mM, PEP 5 mM, pyruvate kinase 1 µg, GTP 0.2 mM, 19 amino acids 0.05 mM, and [^{35}S]Met-tRNA$_f$ 20,000 cpm. The incubation was performed at 37°C for 10 min, and sucrose density gradient, linear 10 to 30 %, centrifugation analysis was carried out at 35,000 rpm for 5 hours at 4°C. Each fraction was put in Bray's solution, and the radioactivity was determined by a scintillation counter. A: without phenomycin (control). B: with phenomycin 10^{-7} M.

inhibition seemed to be lower than that of total protein synthesis. At higher concentration of 10^{-6} M, the degree of inhibition was not significantly increased, suggesting that the tripeptide formation may be also partially affected.

The effect of phenomycin on the ribosomal profile was analyzed by the sucrose density gradient centrifugation. The polysomes were found to decrease, but the 80S and 40S initiation complexes

with Met-$tRNA_f$ were accumulated in the presence of phenomycin 10^{-7} M (Fig. 1). The accumulation of the 80S initiation complex was much more significant than that of the 40S initiation complex. It indicates that the former may result in the latter.

Discussion

The results presented here suggest that initiation of protein synthesis is selectively blocked by phenomycin. In the presence of the antibiotic, the inactive initiation complex is accumulated, and then the initial dipeptide formation is inhibited. It is in accordance with the results that the N-terminal incorporation of valine is more significantly affected by phenomycin than that of methionine.

The above conclusion is also supported by the following observations: (a) Phenomycin blocks globin synthesis more markedly than polyphenylalanine synthesis. (b) The grade of inhibition of globin synthesis is more significant at low Mg^{++} concentration (1 to 3 mM) than at high Mg^{++} concentration (10 mM or higher). (c) The globin synthesis stimulated by addition of the initiation factor is selectively affected by phenomycin. The details will be described elsewhere.

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