THE EFFECTS OF TRIMETHOPRIM AND ERYTHROMYCIN ON POLYSOME METABOLISM IN *ESCHERICHIA COLI*

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(Received for publication November 4, 1977)

Trimethoprim and erythromycin were shown to have different overall effects on *in vivo* polysome metabolism in *Escherichia coli*. In a *rel* A^+–*rel* A pair of strains, trimethoprim treatment induces a reduction of polysome level to a variable extent, similarly to aminoacyl-tRNA deprivation of cells, but persisting polysomes remain dynamic structures in a state of continual turnover. In contrast, erythromycin stabilizes polysome level to a high value in either kind of strain, but maintained polysomes appear as "frozen" structures unable to undergo ribosome translocation.

Numerous studies have been made, at the molecular level, of the mode of action and specificity of trimethoprim and erythromycin, two inhibitors of bacterial protein synthesis[1,2]. Most results agree to indicate that these two drugs act at different steps of polypeptide formation. Indeed, trimethoprim has been shown to be involved in the initiation step by restricting the formylation of methionyl-$tRNA_F$[3,4] whereas erythromycin inhibits the elongation step by binding to the larger subunit of ribosomes[5,6]. Little is known, however, about their overall effects on the metabolism of the protein biosynthetic machinery in the whole cells, *i.e.*, the polysomes.

Such effects were therefore analyzed in the present work. Firstly, the level of polysomes was measured in *in vivo* inhibitor-treated bacteria and compared to that in untreated cells. Secondly, the metabolic stability of polysomes was determined by studying, on the one hand, the process of their decay and, on the other hand, their ability to assemble at the expense of monosomes.

In addition, since it has been shown previously that polysome metabolism in procaryotes under protein synthesis inhibition depends on the allelic state of the *rel* A gene[7~10], the effects of trimethoprim and erythromycin were examined in both a *rel* A^+ (stringent) strain and a *rel* A (relaxed) strain of *E. coli*. The behaviour of polysomes under aminoacyl-tRNA deprivation of the same pair of strains was studied as a control throughout experiments thereafter described.

Materials and Methods

Bacterial strains and culture conditions

The previously described[11] otherwise isogenic pair of *E. coli* strains 10B6 *rel* A^+ and 10B6 *rel* A was used in all experiments. These strains were kindly supplied to us by Dr. S. OSAWA, Hiroshima, Japan. They both are arginine auxotrophs and they harbor a temperature-sensitive valyl-tRNA synthetase (restrictive temperature: 42°C).

Cells were grown exponentially at 30°C under forced aeration in a medium at pH 7.6 containing the following components per liter: 12 g Tris; 2 g KCl; 2 g NH_4Cl; 0.5 g $MgCl_2 \cdot 6H_2O$; 0.02 g Na_2SO_4; 14.7 mg $CaCl_2 \cdot 2H_2O$; 178 mg $Na_2HPO_4 \cdot 2H_2O$; 4 g glucose; 50 mg each of the 20 L-amino acids; 40 mg adenine-HCl; 40 mg cytosine; 40 mg guanine-HCl; 40 mg uracil; 40 mg thymine; 5 mg vitamin B_1.

Glucose starvation

In some experiments, bacteria were grown in the presence of a limiting amount of glucose (0.2 g/liter). Under these conditions, exponential growth was allowed to approximately 4×10^8 cells/ml. At this point, the culture quickly stopped growing due to the exhaustion of the carbon source from the medium.

Preparation of cellular lysates

The method of preparation was similar to that described by KLAGSBRUN and RICH[12]. In a typical experiment, 100 ml of culture were poured over crushed ice in the presence of 100 μg/ml chloramphenicol to prevent ribosome run-off during the extraction procedure[13]. All subsequent operations were carried out at 4°C. Cells were harvested by centrifugation for 8 minutes at $10,000 \times g$. The pellet was resuspended in 12 ml of a sucrose-buffer solution containing 0.5 M RNase-free sucrose, 0.016 M Tris-HCl buffer at pH 8.1, and 0.05 M KCl. Then 1.5 ml of freshly dissolved lysozyme solution (10 mg/ml in sucrose-buffer solution) and 0.3 ml of 10% (w/v) EDTA at pH 8.0 were added to produce protoplasts. The suspension was stirred for 10 minutes and 0.3 ml of 1 M $MgCl_2$ was added to stop lysozyme action. Protoplasts were centrifuged for 6 minutes at $12,000 \times g$ and resuspended in 0.7 ml of freshly prepared lysing medium (0.05 M NH_4Cl, 0.01 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.8) containing 0.5% Brij-58, 0.5% sodium deoxycholate and 5 μg/ml RNase-free DNase. After 10 minutes, the lysate was clarified by centrifugation for 10 minutes at $15,000 \times g$. The supernatant fraction was carefully removed and analyzed by sucrose density gradient centrifugation.

Sucrose gradient analysis

Volumes of 0.2~0.4 ml of clarified lysate were layered onto 15~40% linear sucrose gradients prepared in 0.05 M NH_4Cl, 0.01 M $MgCl_2$ and 0.01 M Tris-HCl, pH 7.8, buffer solution. Gradients were centrifuged in a Beckman (Palo Alto, California) SW 41 Ti rotor for 150 minutes at 39,000 rev/min, then pumped through the continuous-flow cell of a recording spectrophotometer which monitored the optical density at 260 nm.

Quantitative estimation of polysomes

The amount of ribosomal material present in polysomes was determined[11] by measuring the relevant area under the absorbancy tracing after subtraction of the background provided by blank gradients. It was then expressed as a percentage of the total amount of ribosomal material (polysomes + monosomes + ribosomal subunits) taken as 100%.

Antibiotics and chemicals

Chloramphenicol and rifampicin were purchased from Boehringer Mannheim Gmbh. Erythromycin and trimethoprim (2,4-diamino-5[3,4,5-trimethoxybenzyl]-pyrimidine) were from Sigma Chemical Co.

RNase-free sucrose was obtained from Mann Research Labs. All other chemicals used were analytical grade from Merck Co. or Sigma Chemical Co.

Results and Discussion

In a first set of experiments, polysome level was measured in both *rel* A^+ and *rel* A strains under various conditions of protein synthesis inhibition, and was compared to that in exponentially growing cells taken as a control. All measurements were made after a 15-minute period of inhibition. It is shown in Table 1 that, in all cases, polysome level is reduced under trimethoprim treatment (25 μg/ml). The reduction is however much more extensive in the *rel* A^+ strain than in the *rel* A strain. This differential effect can be related to that observed when protein synthesis is blocked by the temperature inactivation at 42°C of valyl-tRNA synthetase, since then a drastic loss of polysomes occurs only in the *rel* A^+ strain. It thus seems that, whatever the strain, trimethoprim-treated cells behave essentially like aminoacyl-tRNA deprived cells as far as polysome maintenance is concerned.

A quite different situation is found in cells treated with erythromycin (1 mg/ml). Indeed, in

Table 1. Percentage of persisting polysomes under various conditions of protein and RNA synthesis inhibition.

Treatment	Strain 10B6 *rel* A$^+$	Strain 10B6 *rel* A
Control at 30°C	46 (100)	56 (100)
Shift to 42°C	17 (37)	56 (100)
Trimethoprim	19 (41)	35 (63)
Erythromycin	56 (122)	65 (116)
Trimethoprim+erythromycin	44 (96)	57 (102)
Rifampicin	0 (0)	<3 (<5)
Shift to 42°C+rifampicin	0 (0)	9 (16)
Trimethoprim+rifampicin	0 (0)	0 (0)
Erythromycin+rifampicin	38 (83)	47 (84)

The percentage of polysomes relative to the control is given in brackets for each strain.

Table 2. Percentage of re-assembled polysomes in cells pre-starved for glucose.

Treatment	Strain 10B6 *rel* A$^+$	Strain 10B6 *rel* A
Glucose starvation	<3 (<6)	<3 (<5)
Control at 30°C	52 (100)	62 (100)
Shift to 42°C	16 (31)	66 (106)
Trimethoprim	23 (44)	57 (92)

The percentage of polysomes relative to the control is given in brackets for each strain.

either strain, not only polysome level is totally maintained but it is even somewhat increased over the control value. One possible explanation for this phenomenon could be that, while polypeptide elongation is blocked in the presence of the drug, a significant amount of previously free ribosomes can still attach to the messenger RNA.

No substantial variation in the proportion of persisting polysomes is observed when either kind of cells are treated simultaneously by trimethoprim and erythromycin. It thus appears that trimethoprim impairs the above-mentioned formation of additional polysomes found in the presence of erythromycin alone.

In order to check whether or not polysomes which are maintained in the absence of protein synthesis are metabolically stable structures, rifampicin, an inhibitor of RNA synthesis[14], was added (250 μg/ml) to cells previously treated for 15 minutes with trimethoprim or erythromycin. Polysome level was then measured after 20 minutes of rifampicin action. It appears, in Table 1, that such treatment results in a complete breakdown of polysomes in cells pre-treated with trimethoprim. Here again, similar results are obtained in valyl-tRNA deprived cells at 42°C. In contrast, polysome level is only slightly affected in cells pre-treated with erythromycin, which indicates that, in the presence of this drug, polysomes behave like "frozen" structures due to the blocking of ribosome translocation along the messenger RNA[15].

Since, on the one hand, polysomes are degraded in trimethoprim-treated cells and, on the other hand, their level is however maintained to a relatively high value, one must assume that they are continuously re-synthesized. To check this point, the process of their assembly under drug treatment was therefore studied. Cultures of either strain were grown exponentially with a limiting amount of glucose then subjected to glucose starvation for 90 minutes. Under these conditions polysomes were

converted into monosomes[8,16]. Such polysome-free cells were then treated with trimethoprim and their polysome content was determined after 15 minutes following glucose readdition. It is shown, in Table 2, that polysomes can re-assemble partially in the *rel* A^+ strain and almost totally in the *rel* A strain. In each case the extent of assembly is similar to that observed in valyl-tRNA deprived cells. One can thus conclude that polysomes which are maintained under trimethoprim treatment are actually dynamic structures which undergo continual turnover, like polysomes of amino acid starved cells[7,8]. However, whether the attachment of ribosomes to messenger RNA, under these conditions, occurs only at physiological sites or at other sites as well, has still to be determined. This problem is currently under investigation in our laboratory.

Acknowledgement

This work was supported in part by a grant from the French National Center for Scientific Research (AI 030085).

References

1) VASQUEZ, D.: Inhibitors of protein synthesis. FEBS Lett. 40: S63~S84, 1974
2) PESTKA, S.: Inhibitors of protein synthesis. "Molecular Mechanisms of Protein Biosynthesis". H. WEISSBACH & S. PESTKA *Eds.*, Academic Press, New York, pp. 467~553, 1977
3) GOLD, L. M. & M. SCHWEIGER: The initiation of T_4 desoxyribonucleic acid dependent β-glucosyltransferase synthesis *in vitro*. J. Biol. Chem. 244: 5100~5104, 1969
4) EISENSTADT, J. & P. LENGYEL: Formylmethionyl-tRNA dependence of amino acid incorporation in extracts of trimethoprim-treated *Escherichia coli*. Science 154: 524~527, 1966
5) TANAKA, S.; T. OTAKA & A. KAJI: Further studies on the mechanism of erythromycin action. Biochim. Biophys. Acta 331: 128~140, 1973
6) FERNADEZ-MUNOZ, R. & D. VASQUEZ: Quantitative binding of ^{14}C-erythromycin A to *E. coli* ribosomes. J. Antibiotics 26: 107~108, 1973
7) RON, E. Z.: Polysome turnover during amino acid starvation in *Escherichia coli*. J. Bacteriol. 108: 263~268, 1971
8) COZZONE, A. J. & P. DONINI: Turnover of polysomes in amino acid starved *Escherichia coli*. J. Mol. Biol. 76: 149~162, 1973
9) CENATIEMPO, Y. & A. J. COZZONE: Evidence for turnover of polysomal structures during aminoacyl-tRNA deprivation in relaxed mutants of *Escherichia coli*. Biochem. Biophys. Res. Commun. 65: 1385~1392, 1975
10) SUBRAHMANYAM, C. S. & H. K. DAS: Amino acid starvation in *Escherichia coli* K12: characteristics of the translation process. J. Bacteriol. 126: 1042~1051, 1976
11) COZZONE, A. J. & G. S. STENT: Movement of ribosomes over messenger RNA in polysomes of stringent and relaxed *Escherichia coli* strains. J. Mol. Biol. 76: 163~179, 1973
12) KLAGSBRUN, M. & A. RICH: *In vitro* synthesis of T_4 bacteriophage head protein using polysomes of T_4 infected *Escherichia coli*. J. Mol. Biol. 48: 421~436, 1970
13) DAVIS, B. D.: Role of subunits in the ribosome cycle. Nature 231: 153~157, 1971
14) GOLDBERG, I. H. & P. A. FRIEDMAN: Antibiotics and nucleic acids. Ann. Rev. Biochem. 40: 775~810, 1971
15) CUNDLIFFE, E. & K. MCQUILLEN: Bacterial protein synthesis: the effects of antibiotics. J. Mol. Biol. 30: 137~146, 1967
16) DRESDEN, M. H. & M. B. HOAGLAND: Polyribosomes of *Escherichia coli*: breakdown during glucose starvation. J. Biol. Chem. 242: 1065~1068, 1967