STRINGENT FACTOR-INDEPENDENT SYNTHESIS OF pppGpp IN *ESCHERICHIA COLI* STRAINS

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Received 3 February 1976

1. Introduction

When growing *E. coli* cells are subjected to unfavourable conditions they accumulate 5′-diphospho-guanosine-3′-diphosphate (ppGpp or MS I) and 5′-tri-phospho-guanosine-3′-diphosphate (pppGpp or MS II). Simultaneously a number of metabolic changes can be observed [1].

Synthesis of guanosine polyphosphates depends on the existence of the stringent factor which is the product of relA gene [2]. As has been proved by in vitro experiments, stringent factor – stimulated by mRNA and by the codon specific uncharged tRNA bound to the ribosomal acceptor site – together with the 70S ribosome synthesizes ppGpp and pppGpp from GDP or GTP substrates [3]. Stringent factor itself is also able to catalyze a slight synthesis of guanosine polyphosphates which may be increased manyfold by adding 20% methanol to the reaction mixture [4].

Here we present a new evidence that the post-ribosomal fraction contains an enzyme which synthesizes pppGpp only. The enzyme is equally found in relA$^+$, relA$^-$ and spoT$^-$ strains. The pppGpp synthesizing activity described by us – when compared and referred to the same cell mass of the stringent factor dependent guanosine polyphosphate synthesizing activity of relA$^+$ strain – was at least 10% of it.

2. Materials and methods

The following strains were used in our experiments: *E. coli* B (prototroph), *E. coli* CP 99 (relA$^+$, spoT$^+$, Arg$^-$, His$^-$, Ser$^-$, B$_1^-$), *E. coli* CP 100 (relA$^-$, spoT$^+$, Arg$^-$, His$^-$, Ser$^-$, B$_1^-$), *E. coli* NF 161 (relA$^+$, spoT$^-$, Arg$^-$, His$^-$, Met$^-$). Strains were tested for their RNA synthesis ([^{3}H]uridine incorporation) and guanosine polyphosphate synthesizing activity during normal and amino acid starved growing conditions.

Culture media contained 15.1 g $Na_2HPO_4 \cdot 12\ H_2O$, 3.0 g KH_2PO_4, 3.0 g NaCl, 2.0 g Na-citrate, 4.0 g casein hydrolysate, 4.0 g yeast extract, 4.0 g glucose per litre. pH was adjusted to 7.2, cells were grown at 37°C and vigorous aeration was achieved by using a horizontal shaker. Growth was stopped at $A_{555\ nm}$ = 2/ml by centrifuging the cells at 5000 *g* at 0°C. Bacterial paste was washed with 0.15 M KCl and stored at −30°C.

2.1. *Preparation of postribosomal supernatant*

Each step was carried out at 0–2°C. Frozen bacterial paste was ground thoroughly with 1.6 parts Al_2O_3 (Sigma type 305), suspended in a buffer containing 0.01 M Tris-HCl pH 7.8, 0.01 M $MgCl_2$, $7 \cdot 10^{-3}$ M β-mercaptoethanol and centrifuged at 25 000 *g*. Supernatant was centrifuged again at 150 000 *g* (Beckman 60Ti rotor) and to the supernatant fraction streptomycin sulphate was added to 1.5% final concentration. The turbid solution was centrifuged at 25 000 *g*. The supernatant was saturated to 75% with ammonium sulphate, pelleted at 25 000 *g* and the precipitate dissolved in a minimal volume of buffer containing 20 mM Tris-HCl pH 7.8, 50 mM KCl, 1.0 mM $MgCl_2$, 0.2 mM EDTA, 5.0% glycerol, 7.0 mM β-mercaptoethanol. The solution was dialyzed overnight against the same buffer and diluted to 2 mg/ml protein content. Dialyzate was assayed for its nucleotide polyphosphate synthesizing capacity.

2.2. *Reaction mixture of the in vitro polyphosphate synthesizing assay system*

Tris-acetate, pH 7.8, 100 mM, NH_4-acetate 20 mM, Mg-acetate 10 mM, K-acetate 10 mM, dithiothreitol 10 mM, GDP or GTP 2.0 mM [γ-^{32}P]ATP 0.16 mM (200 mCi/mM) and 2 μl of enzyme solution in 50 μl final volume. After 3 min preincubation at 37°C the reaction was started by adding [γ-^{32}P]ATP. Incubation period: 10 min at 37°C. Reaction was stopped by adding 5 μl 200 mM EDTA (pH 7.2). 5 μl of this solution was spotted onto polyethyleneimine cellulose (PEI) thin-layer plates (MN-300 PEI, Macherey-Nagel) and dried. Chromatograms were developed in 1.5 M K-phosphate pH 3.4 [5], dried again and autoradiographed. Spots with the corresponding R_f values were cut out and counted by liquid scintillation counting.

^{32}P-labelled authentic pppGpp was prepared from His, Arg-starved CP 99 strain adopting the method of Lazzarini and Cashel [6].

[γ-^{32}P]ATP was prepared by the method of Avron [7]. Nucleoside di- and triphosphates were purchased from REANAL (Budapest), carrier-free ^{32}P isotope from the Isotope Institute of the Hungarian Academy of Sciences.

3. Results

Using the procedure reported in Materials and methods the postribosomal supernatant preparations have a considerable pentaphosphate synthesizing activity which is proportional to the protein concentration (fig.1). The synthesis has an optimum concentration for Mg^{2+} and NH_4^+ in the ranges of 2–20 mM and 10–30 mM, respectively.

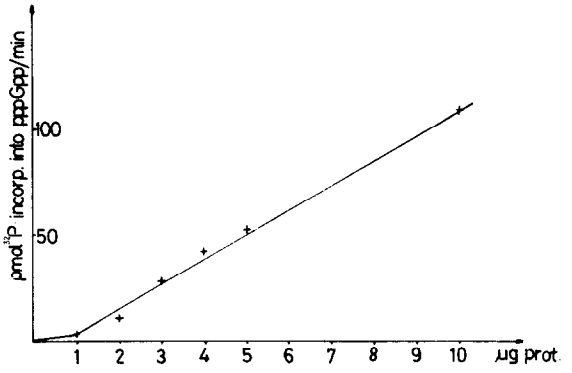

Fig.1. Dependence of pppGpp formation on enzyme concentration. In 50 μl final volume Tris-acetate 100 mM, pH 7.8, NH_4-acetate 20 mM, Mg-acetate 10 mM, K-acetate 10 mM, dithiothreitol 10 mM, GDP 2.0 mM, [γ-^{32}P]ATP 0.16 mM (200 mCi/mmol) and amounts of *E. coli* B enzyme as indicated. Incubation for 10 min at 37°C.

The substrate specificity of the reaction was also demonstrated. Among the nucleoside diphosphates (ADP, UDP, CDP, GDP) tested in our experiments, only GDP can serve as phosphate acceptor. GTP seems to be a much weaker acceptor.

pppGpp synthesized in our system cannot be

Table 1
Stringent factor-independent pppGpp synthesis in *E. coli* strains

Strain	Substrate	^{32}P incorporated into pppGpp (pmoles/μg protein × min)
E. coli B	GDP	12.3
	GTP	0.1
E. coli Cp 99	GDP	16.7
	GTP	0.4
E. coli Cp 100	GDP	11.5
	GTP	0.6
E. coli NF 161	GDP	7.2
	GTP	0.5

Table 2
Effect of antibiotics on stringent factor independent pppGpp synthesizing system

Antibiotics	^{32}P incorporated into pppGpp (pmoles/μg protein × min)
Without antibiotic	11.6
Dimethylsulphoxide 2%	11.8
Thiostrepton 20 μM	11.4
Oxytetracycline 200 μg/ml	9.2
Streptomycin 400 μg/ml	14.2
Kanamycin 400 μg/ml	12.1
Erythromycin 400 μg/ml	11.8

Thiostrepton, oxytetracycline and erythromycin were dissolved in dimethylsulphoxide. Final concentration of dimethylsulphoxide in the reaction mixture: 2%.

distinguished chromatographically from that produced by the authentic *E. coli* strain. Data in table 1 demonstrate that pppGpp is produced with the same efficiency by the postribosomal supernatant fraction of each strain tested, including the relA$^-$ and spoT$^-$ ones.

Ribosome-dependent polyphosphate synthesis can be inhibited by thiostrepton (which inhibits the translocating step in protein synthesis) even at 2.0 μM concentration as well as by oxytetracycline (200 μg/ml) [9].

In table 2 the effect of these and other antibiotics inhibiting protein synthesis is demonstrated. As can be seen, these antibiotics do not inhibit pppGpp synthesis in our system.

4. Discussion

We report here the synthesis of pppGpp from GDP and ATP by the 75% ammonium sulphate precipitable fraction of postribosomal supernatant prepared from *E. coli*. The following data indicate that it is not catalyzed by the stringent factor:

(1) The Mg^{2+} and NH_4^+ ion optimum concentrations differ from those of the ribosomal dependent system [8].

(2) The extract prepared from relA$^-$ mutant is as effective as its relA$^+$ counterpart (table 1) and the relA gene is generally considered to be the structural gene of the stringent factor [2].

An interesting feature of the reaction described here is that pppGpp, and only pppGpp is synthesized from GDP and ATP. The explanation of this phenomenon is not yet clear. One possibility is that ppGpp is the primary product and it is phosphorylated to pppGpp very rapidly. However, this seems unlikely, since the extract prepared from the spoT$^-$ mutant, which is considered to be defective in ppGpp → pppGpp conversion, exerted the same activity as the spoT$^+$ strains [10,11] (table 1).

Another possibility is that first GTP is derived from GDP and ATP and it is converted into pppGpp. This could agree with the new metabolic scheme of MS nucleotide metabolism, according to which ppGpp is derived from the pppGpp (Kari, Török, Travers, personal communication). But, then why is not the added GTP effective in place of GDP?

It is known that the relA$^-$ and relA$^+$ strains have the same basal level of MS nucleotides during balanced growth [6]. The stringent factor-free MS synthesis demonstrated here may have some significance in explaining this phenomenon.

Existence of nucleoside polyphosphates in sporulating *B. subtilis* cells [12], isolation and purification of the ppGpp synthesizing enzyme from culture filtrate of *Streptomyces adephospholyticus* [13] and *Streptomyces morookaensis* [14], occurence of stringent factor independent synthesis pathway in *E. coli* cells, and the presence of nucleotide polyphosphates in eukaryotic cells [15,16] and the fact that mitochondrial and chloroplast preparates are able to produce nucleoside polyphosphates in vitro [17] call attention to the possible metabolism-regulating function of these substances both in prokaryotes and eukaryotes.

Acknowledgements

The authors are very grateful to Drs Lajos Alföldi and Csaba Kari for their advice in preparing this manuscript and to Miss Katalin Havaj and Miss Jolán Papp for excellent technical assistance. This work was partially supported by grants from 'Chinoin' Pharmaceutical Works, Budapest.

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