IN VITRO SYNTHESIS OF GUANOSINE POLYPHOSPHATES IN RAT LIVER MITOCHONDRIAL PREPARATIONS

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Received 23 June 1975

1. Introduction

In 1969 Cashel and Gallant [1] have demonstrated that amino acid starvation in rel$^+$ *E. coli* strains led to the accumulation of guanosine polyphosphates: 5′ diphospho-guanosine-3′ diphosphate (ppGpp) and 5′ triphospho-guanosine-3′ diphosphate (pppGpp) [2,3]. They are thought to be involved in the amino acid control of RNA synthesis.

It was debated whether these substances are restricted to prokaryotes only, or are they present in eukaryotic cells, too? Several laboratories failed to demonstrate the existence of these polyphosphates in eukaryotic cells [4,5]. Later their presence was proved in *Dictyostelium discoideum* [6]. Recently Rhaese [7] described the occurence of ppGpp, pppGpp and other highly phosphorylated substances in mammalian cells in tissue culture (Chinese hamster ovary cells, baby hamster kidney cells and human lung cells).

We demonstrate here the in vitro synthesis of guanosine polyphosphates both in spinach chloroplast and rat liver mitochondrial preparations.

2. Materials and methods

Mitochondria were prepared from the liver of 120–150 g Wistar male rats by the method of Hogeboom et al. [8]. Particles were washed three times in an isolation solution containing sucrose 0.25 M; Tris–HCl 5 mM; EGTA 1 mM; pH 7.5. Preparation and incubation were carried out under semi-sterile conditions, proposed by Kroon [9] and penicillin was present in the incubation mixture. The bacterial contamination (viable count) did not exceed the value permitted for protein synthesizing mitochondrial systems (10^5 cells/ml) [10].

The functional state of particles was checked by respiratory control. Protein content was determined by the modified method of Lowry [11].

2.1 *Guanosine polyphosphate synthesizing assay system*

In a 500 μl final volume: sucrose 150 mM; Tris–HCl 50 mM; KH_2PO_4 2.5 mM; $MgCl_2$ 10 mM; malate 2 mM; glutamate 10 mM; penicillin 200 U/reaction mixture; nucleoside diphosphates and triphosphates (when added) 10 mM; ^{32}P 250 or 50 μCi/reaction mixture (as indicated in the legends); total mitochondrial protein 1.5–1.6 mg/reaction mixture. Final pH was 7.5. Incubation at 30°C, in a horizontal shaker (frequency: 80–120 per min). Incubation period as indicated in the legends.

At appropriate intervals 100 μl samples were taken, 10 μl 4 N formic acid was added and after 10 min at 0°C the precipitate was centrifuged and 5 μl of the clear supernatant was spotted onto polyethyleneimine cellulose (PEI) sheets (Macherey-Nagel and Co., Düren, Polygram cel 300 PEI). Chromatograms were developed in 1.5 M phosphate pH 3.4 [12]. Running distance was 20 or 10 cm (see legends). Spots were located by autoradiography and their radioactivity was determined by liquid scintillation counting.

Crude spinach chloroplast preparation and photophosphorylation was carried out according to the method of Avron [13]. Incubation: 40 min at 15°C. The phosphorylated nucleotides from the deproteinized reaction mixture was adsorbed on Norit A, eluted with alcohol-ammonia solution, lyophilized and redis-

solved in distilled water. 5 μl aliquots were applied onto PEI cellulose sheets.

^{32}P-labeled authentic samples of ppGpp and pppGpp were prepared from CP78 *E. coli* cells under histidine and arginine starvation. Culture media, conditions and the method of ^{32}P-labeled ppGpp, pppGpp synthesis were essentially the same as proposed by Lazzarini and Cashel [14] adopted to the special requirements of CP78 *E. coli* strain.

Nucleoside di- and triphosphates were obtained from REANAL (Budapest). Carrier free ^{32}P from the Isotope Institute of the Hungarian Academy of Sciences.

3. Results

3.1 *Guanosine polyphosphate synthesis in rat liver mitochondrial preparation*

Fig.1 shows a typical experiment with rat liver mitochondria. In the presence of GDP a substantial amount of guanosine polyphosphates was synthesized in an actively phosphorylating mitochondrial preparation.

Some characteristics of the synthesis: Under labeling conditions used in this experiment (cold GDP acceptor and inorganic ^{32}P-orthophosphate, from which labeled ATP and GTP were made by mitochon-

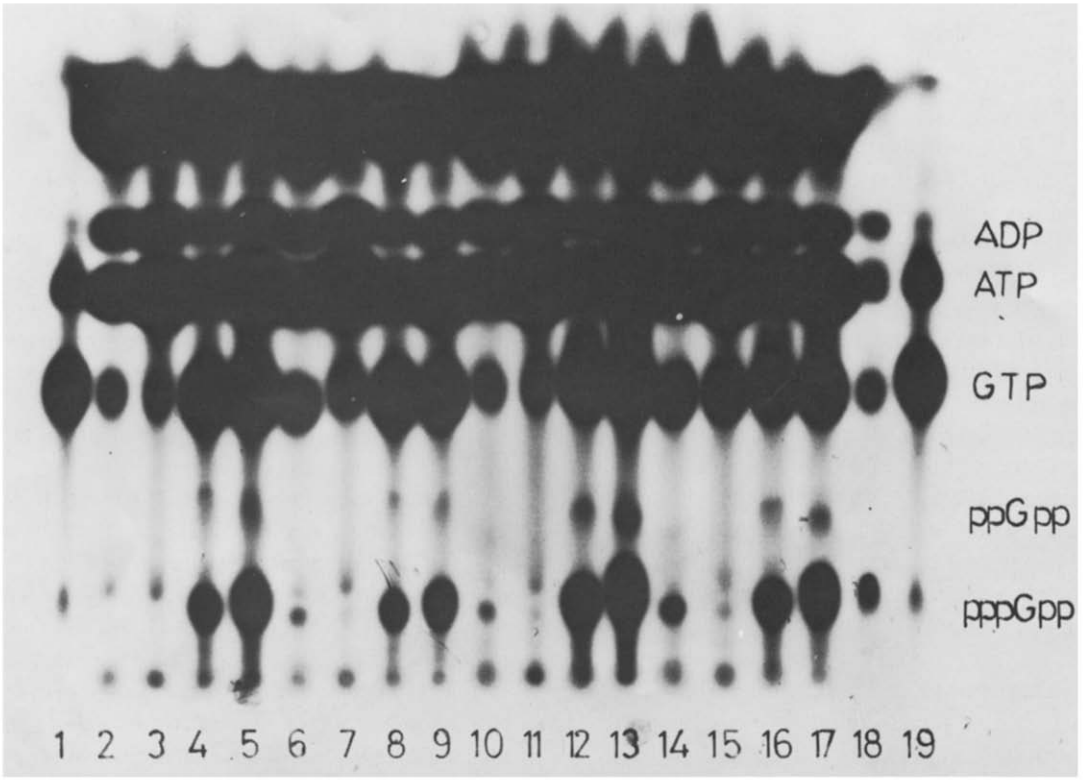

Fig.1. Guanosine polyphosphate synthesis in crude spinach chloroplast and rat liver mitochondrial preparations. Autoradiogram made from PEI cellulose sheet. Samples 1 and 19: material synthesized by spinach chloroplast with added GDP. Sample 18: material synthesized in *E. coli* B cell free system. Samples 2–17: materials derived from mitochondrial system. ^{32}P present: 260 μCi/reaction mixture for mitochondria. Incubation time: for samples 2–9: 20 min, for 10–17: 60 min. For other experimental details see Materials and methods. Additions to the reaction mixture: 2, 10: none; 3, 11: ADP; 4, 12: GDP; 5, 13: GDP+ADP; 6, 14: GMP; 7, 15: ADP+GMP; 8, 16: GDP+glutathione; 9, 17: GDP+ADP+glutathione. Running distance on PEI cellulose: 20 cm.

Table 1
Mitochondrial guanosine polyphosphate synthesis ^{32}P incorporation into guanosine nucleotides

Incubation time (min)	Nucleotides added to the reaction mixture	nmoles ^{32}P incorporated into		
		GTP*	ppGpp*	pppGpp*
20	——		0	0.4
	ADP		0	0.7
	GDP	208	1.1	10.0
	GDP+ADP	258	2.3	24.6
	GMP		0	0.7
	GMP+ADP		0	0.6
60	——		0	0.8
	ADP		0	0.7
	GDP	125	1.5	24.8
	GDP+ADP	278	2.3	42.7
	GMP		0	2.3
	GMP+ADP		0	1.2

* Calculated for 1 ml reaction mixture. Values are taken from the experiment in fig.1. For experimental conditions see Materials and methods and the legend for fig.1. Spots on the PEI sheet were located by autoradiography cut out and their radioactivity determined by liquid scintillation counting.

dria: see autoradiogram in fig.1) pentaphosphates were synthesized preferentially (for quantitative values see table 1). Within a 20 min incubation period approx. 1 per cent of the total ^{32}P activity appeared in the pppGpp, one tenth of this amount in the ppGpp.

The synthesis proceeds longer than 20 min (see table 1).

Substantial amounts of polyphosphates appeared only when GDP acceptor was added to the reaction mixture (see figs.1 and 2).

Other nucleoside-diphosphates (ADP, CDP, UDP) did not serve as ^{32}P acceptor in polyphosphate synthesis (fig.2), however, ADP enhanced, almost doubled, pppGpp labeling when GDP was present, presumably because it created favourable conditions for ATP synthesis. GMP was a very poor acceptor (fig.1). In the presence of GTP, pentaphosphate was also the main reaction product, but the labeling was much lower than that with GDP.

In control samples (without added GDP or in the presence of other nucleotide diphosphates) some labeled pentaphosphates were discernible also (figs. 1 and 2), indicating that active polyphosphate metabolism takes place in this system even if equilibrium is not shifted by addition of high GDP concentration.

In control samples (without GDP: fig.1) spots corresponding to pentaphosphates seem to be split into two spots indicating the presence of two substances with nearly the same R_f values.

3.2 *Guanosine polyphosphate synthesis in chloroplast preparation*

The synthesis of guanosine polyphosphate was also demonstrated in crude spinach chloroplast preparation under conditions favourable for photophosphorylation of GDP to labeled GTP. In fig.1 samples 1 and 19 are preparations made by the chloroplast system. The polyphosphates synthesized in this system comigrate with the authentic *E. coli* pppGpp. It was found in other experiments that this polyphosphate appears only in the presence of GSP. No other nucleoside diphosphate can substitute for it.

4. Discussion

Synthesis of guanosine polyphosphates was demonstrated in eukaryotic cell-free systems: in crude spinach chloroplast and in rat liver mitochondrial preparations.

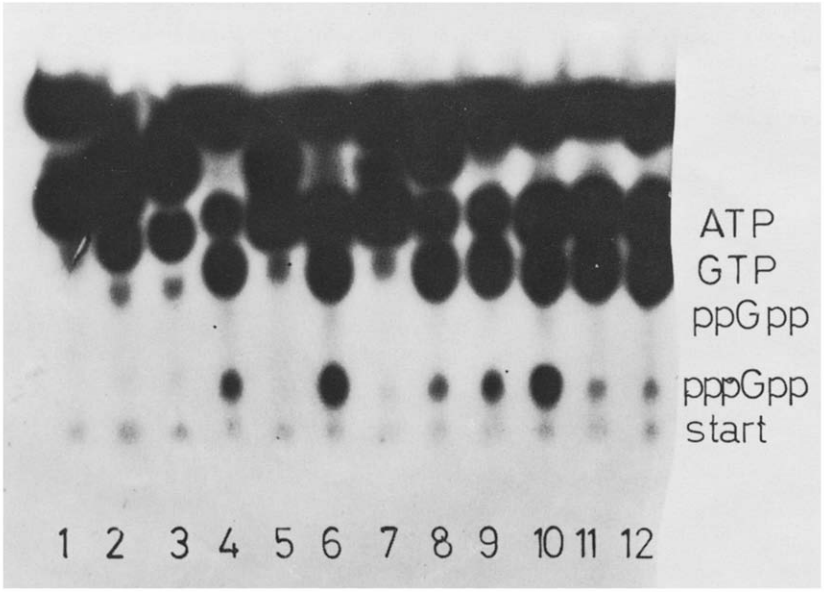

Fig.2. Guanosine polyphosphate synthesis by mitochondrial preparation in the presence of different nucleotides. ^{32}P present in 500 μl reaction mixture: 50 μCi. Incubation period: 20 min. Running distance on PEI cellulose: 10 cm. For other details see Materials and methods. Additions to the reaction mixture: 1: ADP; 2: CDP; 3: UDP; 4: GDP; 5: ADP+CDP; 6: ADP+GDP; 7: ADP+UDP; 8: GDP+CDP; 9: GDP+UDP; 10: GDP+ATP; 11: ADP+GTP; 12: ATP+GTP.

Substantial amounts of polyphosphates were synthesized only in samples containing added GDP or GTP. The quantitative comparison of these two systems is complicated by the different labeling pattern of GDP and GTP. No other nucleotide diphosphates were active in this respect. These observations strongly suggest that the polyphosphates synthesized are guanosine derivatives. The substances could not be distinguished chromatographically from the authentic *E. coli* ppGpp and pppGpp. Further experiments are necessary to validate their identity.

The experimental conditions used exclude the possibility that the observed synthesis is a result of bacterial contamination.

Our results prove that guanosine polyphosphates can be synthesized in eukaryotic cells, too. It involves further questions: have these substances any regulatory functions in eukaryotic cells? If they have, is their function restricted to mitochondria only or do they interfere with other cellular functions? Are they synthesized inside the mitochondria or other subcellular organelles are involved in it? Our results to date support the first supposition.

Acknowledgements

The authors are very grateful to Drs Lajos Alföldi and István Török for providing *E. coli* CP78 and for critical discussions, to Miss Judit Jurina and Miss Katalin Havaj for excellent technical assistance. This work was partially supported by grants from 'Chinoin' Pharmaceutical Works, Budapest.

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