Identification of the Synthesis of Guanosine Tetraphosphate (MS I) as Insertion of a Pyrophosphoryl Group into the 3'-Position in Guanosine 5'-Diphosphate

(E. coli/stringent/relaxed control/phosphate transfer)

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ABSTRACT The phosphate transfer system of Haseltine et al., consisting of a ribosomal wash obtained from a stringent strain of Escherichia coli, washed ribosomes, GTP, and ATP, was used to prepare large quantities of guanosine tetra- and pentaphosphates, the magic spot compounds MS I and MS II of Cashel and Gallant. In our hands, the Haseltine et al. system yielded predominantly guanosine tetrathosphate, ppGpp. This system was used exclusively in the described experiments, with ATP labeled with 32P in the α- and γ-positions as donor. The β-label was found to produce a ppGpp and the γ-label a ppGpp. Furthermore, [γ-32P]ATP yielded ppGpp in a 4H : 32P ratio of 1 : 1. The results indicate a transfer of the terminal pyrophosphoryl group of ATP as a unit.

The position of the transferred pyrophosphoryl was assayed for by preparation of pGp from ppGpp with Zn++-activated inorganic pyrophosphatase from yeast. The pGp was then assayed with 3'-nucleotidase, which liberated practically all the labeled phosphate. This result indicates that the phosphate transfer from ATP to GDP yields guanosine 5'-diphosphate-3'-diphosphate.

New aspects of the stringency problem (1) have been revealed through the discovery by Haseltine et al. (2) of a factor present in the ribosomal wash of stringent, but not of relaxed, Escherichia coli. This factor takes part in the catalysis of transfer of two phosphates from ATP to GDP or GTP, whereby, on addition of two phosphates into 2'- or 3'-position, guanosine tetra- and pentaphosphate (3), the “magic spot” compounds MS I and MS II, respectively, of Cashel and Gallant (1), are produced. The enzyme system described by Haseltine et al. (2) is complex, insofar as the transfer activity is dependent on the 0.5 M NH4Cl ribosomal wash, as well as on the presence of ribosomes and elongation factor G (EF-G), and is blocked by fusidic acid, a specific inhibitor of the EF-G-linked translocation step in protein synthesis.

This result aroused our interest because it may involve, in the stringency effect, the translocation with which this laboratory has been concerned for some time (4). In the present report we have exploited the availability of a relatively easy method (2) of obtaining sizeable quantities of the “magic spot” guanosine polyphosphates for analysis of the mechanism of the transfer reaction. By obtaining transfer products from ATP marked at various positions, it has been possible to show: (i) that synthesis from ATP is due to pyrophosphoryl transfer, and (ii) that this transfer yields the 3'-pyrophosphoryl derivative of guanosine 5'-polyphosphates.

MATERIALS

The stringent strain used for preparation of the enzyme system was E. coli K-19 (strept, thr-, leu-, B1, gal+, lac+, F+); it was kindly supplied by Dr. Nortin Zinder and checked for stringency by Dr. Peter Model. The organism was grown in bulk at the Oak Ridge National Laboratory, with the most valuable cooperation of Dr. Novelli's group in the Biology Division. The organism was prepared through to ribosomes, from which the enzyme system was prepared in this laboratory. The NH4Cl-washed ribosomes and 0.5 M NH4Cl ribosomal wash were prepared as described (2).

Yeast inorganic pyrophosphatase (840 units/mg) and 3'-nucleotidase from Rye Grass (14 units/mg) were purchased from Sigma. Carrier-free 32P, [α-32P]GTP, and [γ-32P]ATP were obtained from Schwars/Mann. ATP, AMP, GTP, and 5'-, 3', and 2'-GMPs were obtained from P-L Biochemicals. Polyethyleneimine (PEI)-cellulose thin-layer sheets came from Brinkmann Instruments. [γ-32P]ATP was a generous gift from Mr. Owen Griffith. [β-32P]ATP was synthesized by the following method: [β-32P]ADP was formed by myokinase from [γ-32P]ATP and 5'-AMP, and was purified on a DEAE-cellulose column. It was then phosphorylated to [β-32P]ATP by pyruvate kinase and phosphoenolpyruvate.

RESULTS AND DISCUSSION

Since the aim of the present study was to explore the transfer mechanism of phosphate from ATP to GTP and/or GDP, we set up the system as described (2) and followed the course of reaction using GTP and ATP as the reactants. As shown in Fig. 1, the presently used system includes ribosomes and EF-G, and the GTP is rapidly converted to GDP. The figure shows that, in the beginning, with mostly GTP present, the reaction starts with relatively small amounts of guanosine...
pentaphosphate being formed. However, as soon as larger amounts of GDP appear, the latter competes successfully for ATP as donor and, after incubation for 20 min, almost exclusively guanosine tetraphosphate is formed. Since a preponderance of GDP as phosphate acceptor has been the rule in our set-up and ppGpp was easy to obtain in quantity, it was decided to concentrate on the mechanism of its synthesis. In view of the obvious parallel (2) between the phosphate transfer to various guanosine phosphate acceptors, we consider it as a probe for the general mechanism of the reaction between ATP and guanosine polyphosphates in this system.

**The mode of phosphate transfer from ATP to GDP**

In order to perform experiments designed to identify the mechanism by which phosphate is transferred from the donor ATP, it was necessary to obtain ATP labeled with $^{32}$P in the $\beta$- and $\gamma$-positions. To produce a pyrophosphoryl transfer by ATP, two types of enzymatic reaction are known: either the two phosphates may be transferred one-by-one, as for example in the pyrophosphorylation of mevalonic acid (6, 7), or they may be transferred as a pyrophosphoryl group, as is realized in the synthesis of pyrophosphoryl 1'-ribose-5'-phosphate. In the latter reaction, Khorana et al. (8) showed the $\beta$-phosphate of ATP to be inserted into the acceptor in the 1'-position of ribose 5'-phosphate:

$$\text{pppA} + \text{ribose-5'}-P \rightarrow \text{pA} + \text{pp-1'-ribose-5'}-P$$  \[1\]

On the other hand, in mevalonate phosphorylation through one-by-one transfer, both phosphates derive from $\gamma$-phosphates of two ATPs:

$$2\text{pppA} + \text{mevalonate} \rightarrow 2\text{pA} + \text{pp-mevalonate}$$  \[2\]

To decide which of the two mechanisms was operating here, in the first experiment [H]GTP was used as acceptor and [$\gamma$-$^{32}$P]ATP as donor. A ratio of 1:1 was obtained on analysis of the ratio H:$^{32}$P in ppGpp (Fig. 2). This result indicated pyrophosphoryl transfer, since single transfer should have resulted in a ratio of 1:2. A second method was applied to

![Fig. 1. Kinetics of ppGpp and pppGpp synthesis. NH$_4$Cl-washed ribosomes (145 $\mu$g, 2 $A_{260}$ units) and ribosomal wash (39 $\mu$g) were incubated at 37$^\circ$C in 50 $\mu$l of 40 mM Tris-acetate (pH 7.8), 2 mM dithiothreitol, 10 mM MgCl$_2$, 100 mM NH$_4$Cl, 0.43 mM [a-$^{32}$P]GTP (23 Ci/mol), and 3.6 mM ATP. At various time points, 5 $\mu$l of reaction mixture was withdrawn and mixed with 5 $\mu$l of 3.5% formic acid. After removal of the precipitated ribosomes by centrifugation, 2 $\mu$l of the supernatant fraction was applied to PEI-cellulose plates, which were then dried, washed with absolute methanol, and developed with 1.5 M K$_2$HPO$_4$ (pH 3.4) (3). The developed plates were dried and radioautographed. Regions corresponding to GDP, GTP, ppGpp, and pppGpp were then cut out and counted in a Packard scintillation counter with Bray's solution (5).](image1)

![Fig. 2. $^1$H:$^3$P ratio in double-labeled ppGpp. The double-labeled [H]ppppGpp was synthesized in the in vitro system with [H]GTP and [$\gamma$-$^{32}$P]ATP, in 50 $\mu$l containing: 40 mM Tris-acetate (pH 7.8), 1 mM dithiothreitol, 10 mM Mg(OAc)$_2$, 25 mM NH$_4$OAc, 0.55 mM [H]GTP (91 Ci/mol), 1.6 mM [$\gamma$-$^{32}$P]ATP (110 Ci/mol), and 100 $\mu$g of ribosomes. Incubation was at 37$^\circ$C for 1 hr. The reaction was stopped by the addition of 1 $\mu$l of 88% formic acid. The precipitated ribosomes were removed by centrifugation, and the resulting supernatant fraction was diluted with 1.0 ml of 50 mM triethylammonium bicarbonate buffer (pH 7.7). The neutralized sample was then applied to a 1-ml DEAE-cellulose column that had been equilibrated with the same buffer. The column was washed with the following concentrations of triethylammonium bicarbonate (pH 7.7): 1.0 ml of 50 mM, 2 ml of 100 mM, 6 ml of 150 mM, 10 ml of 200 mM, and 4 ml of 300 mM. Fractions of 1 ml were collected and counted for the two labels in a Packard scintillation counter. In this system, by stepwise elution, ATP and GTP were eluted with 0.2 M buffer, and ppGpp with 0.3 M buffer. For isolation of ppGpp, the 0.3 M eluate was lyophilized to remove excess salt, and dissolved in a small amount of water. As thus isolated, ppGpp shows only one spot on radioautography with the two-dimensional chromatography system: (1) 3.3 M ammonium formate + 4.2% boric acid brought with NH$_4$OH to pH 7; (2) 1.5 M KH$_2$PO$_4$ (pH 3.4) (3).](image2)
confirm the pyrophosphoryl transfer by use of a $^{32}$P-label in the donor ATP in either the $\beta$- or $\gamma$-positions (Fig. 3), obtained as described in Materials, followed by analysis of the products by acid hydrolysis for the liberation or retention of $^{32}$P (Fig. 4). Eq. 1 indicates that in our case, by phosphoryl transfer from $\beta$-label in ATP, the $^{32}$P would transfer directly to the 2'$\prime$- or 3'$\prime$-position on the ribose in guanosine polynucleotide and would be stable to mild acid hydrolysis. However, the $\gamma$-$^{32}$P of ATP would move into the outer, acid-labile phosphate of the pyrophosphoryl group and be removed by the acid. The incubation at $37^o$ for 30 min with 1 N HCl was found to split only the 3'$\prime$-pyrophosphate, since it is more sensitive to acid than the 5'$\prime$-pyrophosphoryl group. Accordingly, Fig. 4 indicates that such hydrolysis of ppGpp yields only ppGp, and, furthermore, that with $\gamma$-labeled ATP as donor, all label is hydrolyzed to P$_1$ and none remains with the nucleotide. However, with $\beta$-label in the donor ATP, the reverse is true: practically no $^{32}$P is released as P$_1$ but all label remains with ppGp; thus ppGp is formed on hydrolysis. Similar results were obtained when ppGpp was hydrolyzed for 24 hr at $37^o$ in 1/3 N KOH.

These are the results to be expected with pyrophosphoryl transfer from the example given in Eq. 1; with $\gamma$-label in ATP, on transfer, the label appears in the outer phosphate of ppGpp and is released with acid or alkali. From the $\beta$-label it moves into the inner position in ppGpp and becomes acid-stable. Thus, both methods confirm the joint transfer of the two terminal phosphates from ATP to GDP. Indications for a pyrophosphoryl transfer have also been obtained by Cashel (personal communication).

\textbf{Fig. 3.} Synthesis of ppGpp labeled with $^{32}$P in various positions. The label was introduced by use of the following reactants: $A$. 1 mM GTP, 1 mM $[\beta^{32}$P]ATP (60 Ci/mol), and $B$, 1 mM ATP, and 1 mM $[\gamma^{32}$P]ATP (190 Ci/mol). Reaction mixtures contained in 50 $\mu$I: 20 mM Tris-acetate (pH 7.8), 2 mM dithiothreitol, 10 mM Mg(OAc)$_2$, 145 $\mu$g of washed ribosomes, and 39 $\mu$g of 0.5 M NH$_4$Cl ribosomal wash, which contained the necessary EF-G. Incubations were performed at $37^o$ for 1 hr and stopped by chilling and addition of 1 $\mu$l of 88% formic acid. The precipitate containing the ribosomes was removed by centrifugation, and 1 $\mu$l of the supernatant was chromatographed on PEI-cellulose thin-layer sheets using 1.5 M KH$_2$PO$_4$ (pH 3.4) as developing solvent. The chromatograms were then scanned in a Varian radio scanner. Marker nucleotides, indicated by arrows, were visualized under ultraviolet light. The transfer reactions realized in the two sets of experiments are formulated in the following equations: $(A)$ ppG + $\gamma$ppA $\rightarrow$ ppGpp + pA; $(B)$ ppG + $\beta$ppA $\rightarrow$ ppGpp + pA.

\textbf{Identification of the position in which the pyrophosphoryl group is attached}

Coenzyme A and active sulfate each contain an additional phosphate attached to the adenylic acid residue. In both cases, the additional phosphate was determined to be in the 3'$\prime$-position by use of 3'$\prime$-nucleotidase prepared from Rye Grass according to Shuster and Kaplan (9). This enzyme was used by Wang et al. (10) in the case of CoA and by Robbins and Lipmann (11) for analysis of active sulfate. In the following, it will be shown that 3'$\prime$-nucleotidase can also be used for positioning the phosphoryl group in ppGpp. As shown by Shuster and Kaplan (9), the enzyme is a nucleotide 3'$\prime$-phosphatase; in particular, it hydrolyzes guanosine 3'$\prime$-phosphate with relative ease, although not as well as adenosine 3'$\prime$-phosphate. We tried the 3'$\prime$-nucleotidase used here and found it to be quite active with guanosine-3'$\prime$-P and practically inactive with guanosine 2'$\prime$-P. It did not hydrolyze ppGp, and, therefore, the terminal phosphates were first removed with crystalline inorganic pyrophosphatase. This enzyme had been found to hydrolyze nucleotide polyphosphates when it was activated with Zn$^{++}$ instead of Mg$^{++}$ (12). As shown in the preceding section, when ATP with $^{32}$P in the $\beta$-position is used as the donor, ppGp is formed, and it was used as the substrate for the following experiments.

\textbf{Fig. 4.} Stepwise acid hydrolysis of variously labeled ppGpp. ppGpp Preparations were labeled with $^{32}$P (labeled as in Fig. 3 and isolated as in Fig. 2) as follows: $(A)$ ppGp (23,000 cpm), and $(B)$ ppGP$^*$ (27,000 cpm). For acid hydrolysis they were incubated in 10 $\mu$l of 1 N HCl at $37^o$ for 30 min. Hydrolysis was stopped by chilling in ice and the addition of 10 $\mu$l of 1 M Tris-HCl (pH 10). The neutralized samples were applied to PEI-cellulose sheets and excess salts were washed away by immersion in absolute methanol (15). Thin-layer chromatography sheets were developed in 0.75 M KH$_2$PO$_4$ (pH 3.4). The radioactivity of the sheets was scanned with a Varian aerograph Berthold radioscanner. Under the conditions of this experiment the short 1 N HCl exposure was expected to convert ppGp to ppGP; $\rightarrow$ ppGp + p$_1$, whereas longer hydrolysis will further convert ppGp to pGp (Fig. 5B).
Fig. 5. Hydrolysis of ppGpp to pGp by yeast inorganic pyrophosphatase and by acid. (A) ppGpp (1.2 x 10^6 cpm) was hydrolyzed in 100 ml containing 10 mM Tris-HCl (pH 7), 0.4 mM Zn(OAc)_2, and 10 units of yeast inorganic pyrophosphatase at 37° for 12 min. The reaction was stopped by chilling on ice and subsequent application to PEI-cellulose thin-layer sheets. The sheets were then developed with 4 M Na-formate (pH 3.4). After air-drying of the developed chromatogram, salts were removed by thorough washing with absolute methanol (15). The chromatogram was then scanned with the Varian radioscanner. The desalted chromatogram was radioautographed, and zones corresponding to pGp were cut out and eluted with 2 ml of 0.5 M triethylammonium bicarbonate (pH 8.0). After two more elutions with 1 ml (each) of triethylammonium salts, the combined eluates were lyophilized. As thus isolated, pGp was shown to be homogeneous in two different systems of development on PEI-cellulose: 1.6 M LiCl (R_F = 0.51), and 0.75 M KH_2PO_4 (pH 3.4) (R_F = 0.61). (B) ppGpp (2.3 x 10^6 cpm) was hydrolyzed in 100 ml of 1 N HCl for 16.5 hr at 37°. Hydrolysis was stopped by neutralization with 1 M Tris-HCl (pH 10). The neutralized sample was then applied to PEI-cellulose sheets. Excess salts were removed by thorough washing with absolute methanol. The chromatogram was developed and the sample was isolated as described in A.

Relatively large amounts of yeast inorganic pyrophosphatase and a short incubation yielded the desired pGp (Fig. 5A). For comparison, the analogous pGp was obtained by prolonged acid hydrolysis (Fig. 5B). Here, however, the exposure to acid was expected to cause equilibration between the 3' and 2' positions (13, 14). Both compounds were isolated and exposed to 3'-nucleotidase. The result is shown in Fig. 6. The enzyme released practically 100% of the 32P as P_i from the product of enzymatic hydrolysis (upper curve). In contrast, acid hydrolysis yielded a product from which the enzyme split just about half as much label. This result was to be expected, in view of the equilibration by acid, which left only half of the label available for 3'-nucleotidase hydrolysis.

Thus, the position of the transferred pyrophosphoryl group in guanosine tetraphosphate is defined as 3'. Since both GTP and GDP are pyrophosphoryl acceptors (compare Fig. 1) with this enzyme system, the guanosine penta-... as well as the tetrathosphates, that we have tested here are expected to be synthesized by transfer to the 3'-position.

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