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Effect of Aminoacyl Transfer Ribonucleic Acid on Competition between Guanosine 5′-Triphosphate and Guanosine 5′-Diphosphate for Binding to a Polypeptide Chain Elongation Factor from *Escherichia coli**

David Cooper† and Julian Gordon

ABSTRACT: The polypeptide chain elongation factor preparation referred to as T (an undissociated combination of T_u and T_s) exhibited preferential binding of guanosine 5′-diphosphate, even in the presence of a large excess of guanosine 5′-triphosphate, when tested for retention on Millipore filters. Previously published data on binding of guanosine 5′-triphosphate using this assay is now shown to be attributable to the preferential binding of the [^{3}H]guanosine 5′-diphosphate present in the [^{3}H]guanosine 5′-triphosphate preparation. Removal of this guanosine 5′-diphosphate with a guanosine 5′-triphosphate regenerating system considerably reduced the level of bound nucleoside phosphate. Nonradioactive guanosine 5′-triphosphate did not compete with Millipore-bindable [^{3}H]guanosine 5′-diphosphate. Addition of aminoacyl transfer ribonucleic acid reversed this preference for guanosine 5′-diphosphate.

It is now clear that the mechanism by which aminoacyl-tRNA binds to *Escherichia coli* ribosomes during polypeptide synthesis involves the polypeptide chain elongation factor T of Nishizuka and Lipmann (1966), which was subsequently resolved into two factors, T_u and T_s, by Lucas-Lenard and Lipmann (1966). This binding also requires GTP (Ravel *et al.*, 1967, 1968; Lucas-Lenard and Haenni, 1968; Ertel *et al.*, 1968b). The binding of aminoacyl-tRNA to ribosomes was originally proposed to proceed in two steps, on the basis of comparison of Millipore binding assays, which detected the first step (T–GTP); and gel filtration assays with Sephadex G-50 which detected a ternary complex (T–GTP–aminoacyl-tRNA), characterized by its inability to bind to the Millipore (Gordon, 1967, 1968; Ravel *et al.*, 1968; Ertel *et al.*, 1968a). More recent studies have suggested that the subfraction T_u is the acceptor for nucleoside phosphate (Ertel *et al.*, 1968a; Ravel *et al.*, 1969). However, in the experiments described in this paper, T_u and T_s were not dealt with separately, so we retain the terminology T factor for convenience.

More detailed experiments have now shown that the Millipore assay selectively detects the binding of the trace of GDP preexisting in commercial preparations of GTP, and this preference was overcome by the addition of aminoacyl-tRNA. These experiments are the subject of this communication.

Materials and Methods

GTP was supplied by P-L Biochemicals, Inc., and repurified essentially by the method of Moffatt (1964). A 0–0.4 M triethylammonium bicarbonate gradient (pH 7.5) was used to elute the nucleotides from a DEAE-cellulose column (Whatman DE-11). Fractions containing GTP were identified by thin-layer chromatography as described below, pooled, and lyophilized.

[^{3}H]GTP (lithium salt, specific activity 1.4 Ci/mmole) and [^{3}H]GDP (lithium salt, specific activity 1.27 Ci/mmole) were

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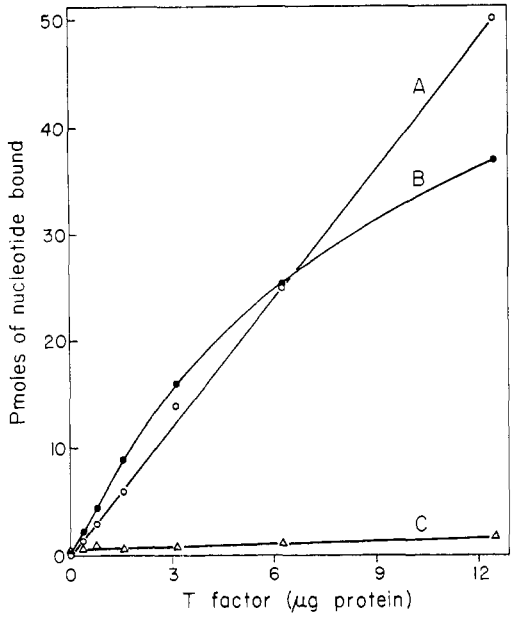

FIGURE 1: Binding of radioactive nucleotides to T factor assayed by Millipore binding. Each reaction mixture contained, in addition to the standard components: (A) 400 pmoles of [^{3}H]GDP, (B) 700 pmoles of [^{3}H]GTP, and (C) 740 pmoles of [γ-^{32}P]GTP. Reaction was continued for 2 min at 30°.

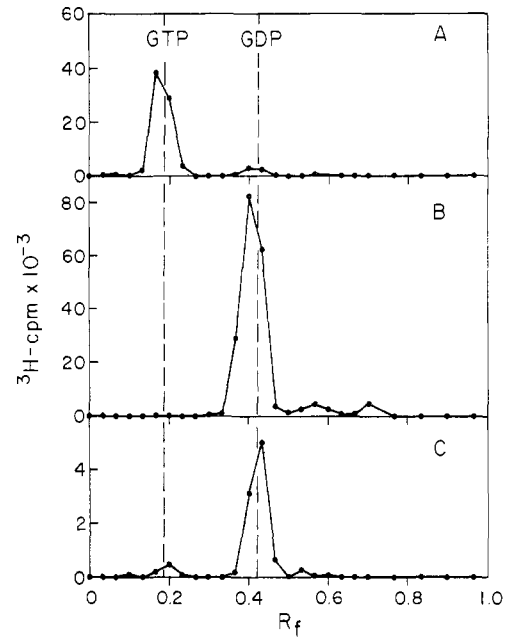

FIGURE 2: Chromatographic analysis of samples on polyethyleneimine thin-layer plates. (A) Commercial GTP, (B) commercial GDP, and (C) eluate from Millipore after binding assay with GTP sample as in part A used as substrate.

supplied by Schwarz BioResearch, Inc. [γ-^{32}P]GTP was from International Chemical and Nuclear Corp. The level of $^{32}P_i$ in the latter was reduced to 0.3% by stepwise chromatography on DEAE-cellulose, modified from the procedure of Moffatt (1964).

L-[^{14}C]Phenylalanine (325 mCi/mmole), supplied by Schwarz BioResearch, Inc., was used to charge tRNA (General Biochemicals, Inc.) of *Escherichia coli* B by the method of Conway (1964). The product contained 500 pmoles of phenylalanine/mg of RNA. Radioactivity from [^{14}C]-phenylalanine was counted in a Nuclear-Chicago low-background gas-flow counter at approximately 20% counting efficiency.

Nucleotides were chromatographed on polyethyleneimine cellulose impregnated MN 300 thin-layer plates (Brinkmann Instruments, Inc.) with 1 M potassium phosphate (pH 3.4) as developing solvent (Cashel *et al.*, 1969). The spots were identified by viewing chromatograms in ultraviolet light, or by cutting them in 5-mm sections and determining the radioactivity of each section with 5 ml of toluene–Liquifluor (Pilot Chemicals, Inc.) in a Nuclear-Chicago scintillation counter. Radioactivity bound to the Millipore was transferred directly to a polyethyleneimine thin-layer plate by elution with 50 mM EDTA (pH 7.5) using a technique similar to that of Laskowski (1967). Over 90% of the radioactivity (assessed from a duplicate) was transferred in this way. Unlabeled GDP and GTP (0.01 μmole of each) were added before development of the chromatogram.

E. coli B used for the preparation of polypeptide chain elongation factors T and G (Nishizuka and Lipmann, 1966) was grown at 37° with vigorous aeration in a Biogen (American Sterilizer Co.). The medium contained, per liter, 10 g of glucose, 8 g of Difco nutrient broth, and 5 g of Difco yeast extract supplemented with the salts solution of Schaechter *et al.* (1958). Cells were continuously harvested in logarithmic phase (optical density of 3.5 at 450 mμ in a Zeiss Model PHQ II spectrophotometer) and were not frozen before use.

Details of the methods used to purify the ribosomes and factors T and G are to be published separately (Gordon, 1969). In summary, the steps were: centrifugation of the cell homogenate at 30,000*g*, and extraction of nucleic acids and ribosomes from the supernatant fraction by a polyethylene glycol–Dextran phase system (Albertsson, 1960; Alberts, 1967); separation of T factor from G factor by ammonium sulfate fractionation; DEAE-cellulose column chromatography; hydroxylapatite column chromatography (Parmeggiani, 1968); and preparative polyacrylamide gel electrophoresis (Parmeggiani, 1968). Both factors T and G showed single bands on analytical polyacrylamide gel electrophoresis. The factors T_u and T_s (Lucas-Lenard and Lipmann, 1966) were not studied separately in the present work.

The Millipore assay for binding of nucleotide to T factor was essentially as before (Gordon, 1968). The reaction mixtures contained in a total volume of 0.105 ml: 50 mM Tris-HCl (pH 7.4), 160 mM ammonium chloride, 10 mM magnesium chloride, 12 mM dithiothreitol, nucleotides, tRNA, and factor as specified. Reactions were started by the addition of 5 μl of T factor solution, and terminated after 30 sec at 0° (when the reaction had already gone to completion) or as otherwise specified. Reaction mixtures were filtered and washed as previously described, and radioactivity was assayed in the filters by scintillation counting. The Millipore filters used in this assay (HA 0.45 μ pore size) were washed with NaEDTA (pH 8) and then with water before use. All buffer solutions were Millipore filtered to remove particles of dust which caused variable blanks.

GTP hydrolysis and poly U directed phenylalanine poly-

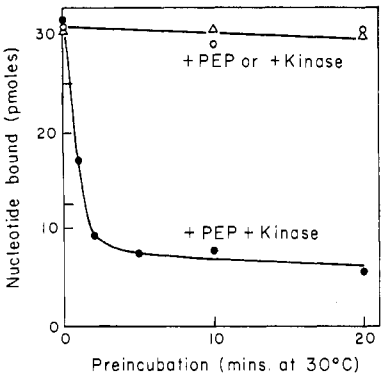

FIGURE 3: Effect of preincubation of [^{3}H]GTP with triphosphate-regenerating system on the amount of nucleotide subsequently bound to T factor. The standard reaction mixture (before addition of enzyme) was supplemented with either phosphoenolpyruvate (5 mM) or 50 μg of pyruvate kinase (upper curve), or both together (lower curve) during preincubation. Each tube contained 700 pmoles of [^{3}H]GTP. After preincubation, the reaction mixtures were cooled in an ice bath, and 12.5 μg of T factor was added. After 30 sec they were filtered and washed.

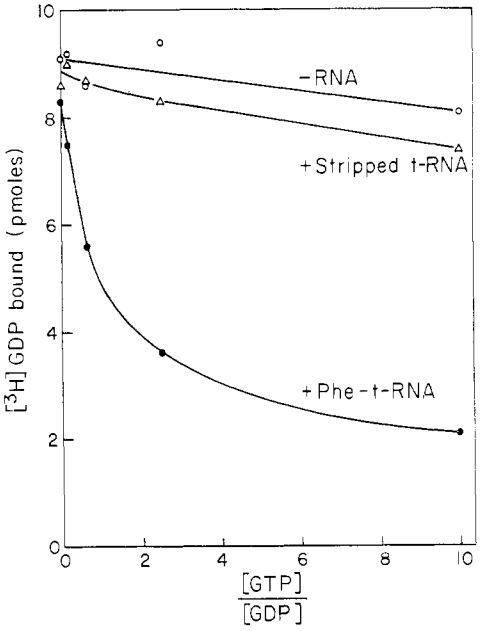

FIGURE 4: Effect of phenylalanyl-tRNA and GTP on the binding of GDP to T factor. Each reaction mixture contained 100 pmoles of [^{3}H]GDP and purified unlabeled GTP as shown by the abscissa. Where indicated, 75 μg of stripped tRNA or 73 μg of [^{14}C]phenylalanyl-tRNA (containing 37 pmoles of phenylalanine) was also added. The binding was started by the addition of 3 μg of T factor and continued for 1.5 min at 30° before filtration.

merization were assayed by the method of Nishizuka and Lipmann (1966) using dithiothreitol instead of mercaptoethanol.

Phosphoenolpyruvate (tricyclohexylammonium salt) and phosphoenolpyruvate kinase (EC 2.7.1.40) were supplied by Boehringer Mannheim Corp.

Results

The T factor used in this study was active in complementing factor G for phenylalanine polymerization. In the standard assay, 1 μg of T resulted in the polymerization of approximately 30 pmoles of phenylalanine. It therefore contained both factors T_u and T_s which are necessary for amino acid polymerization. The T factor was also free of GTPase activity (less than 0.2 pmole of P_i was released from 1000 pmoles of GTP by 10 μg of T factor in 1 min at 30°).

Figure 1 illustrates an experiment in which labeled GDP or GTP was incubated with various amounts of T factor. The binding of tritium-labeled GDP by Millipore filter was linear with protein concentration, but when [^{3}H]GTP was used there was a detectable leveling of the amount of tritium bound. Very little ^{32}P was retained when [γ-^{32}P]GTP was used, suggesting that much of the nucleotide bound to T factor was not GTP.

To identify the nucleotide retained on the Millipore filter, [^{3}H]GTP was incubated with T factor and the reaction mixture was filtered and washed in the usual way. The bound radioactivity was then analyzed by thin-layer chromatography after elution from the Millipore filter (see Methods section). Figure 2 shows that although [^{3}H]GTP was added to the reaction mixture, 90% of the radioactivity bound chromatographed as GDP and only 10% as GTP. Figure 2A shows that the commercial [^{3}H]GTP contained [^{3}H]GDP as a contaminant. Although GDP comprised only 10% of the nucleotide present in tritiated GTP added to the binding reaction mixture, the T factor showed a remarkably high selectivity for GDP. When reaction mixtures containing T factor and [^{3}H]GTP and no aminoacyl-tRNA were passed through Sephadex G-50 columns, relatively small amounts of bound radioactivity were obtained (for direct comparison of the Millipore and Sephadex assays, see Gordon, 1968). However, an analysis similar to that of Figure 2 of the complex that survived passage through Sephadex G-50 also showed a strong preferential binding of the contaminating GDP.

Experiments were also performed in which the tritiated GTP was preincubated with a nucleoside triphosphate regenerating system before the addition of T factor. Figure 3 shows that this procedure reduced by 80% the amount of tritiated nucleotide bound to the T factor in the Millipore assay. Chromatography showed that the tritium binding, which could not be eliminated by preincubation with the regenerating system, was largely GTP. The lower level of [γ-^{32}P]GTP bound in the experiment of Figure 1 was probably due to the presence of nonradioactive GDP. The T factor is therefore highly selective in preferentially binding GDP and little GTP binding can be detected by the Millipore filtration assay. This experiment also eliminates the possibility that GTP, which might have initially bound to the T factor, was being hydrolyzed as the complex was filtered on the Millipore.

It was found with the electrophoretically homogeneous T factor preparation used here, as reported earlier by Gordon (1968), Ravel *et al.* (1968), and Ertel *et al.* (1968a,b), that addition of aminoacyl-tRNA to reaction mixtures containing tritiated GTP resulted in a reduction of label retained on the Millipore filter. The requirement for GTP in this process is shown in Figure 4. The GTP was repurified by DEAE-cellulose chromatography to remove contaminating GDP. The graph shows that unlabeled GTP was not effective in displacing labeled GDP bound to T factor, whereas both GTP and

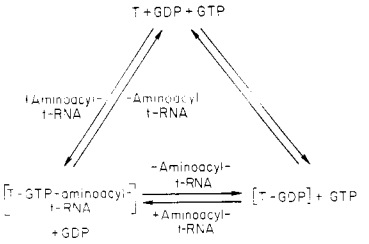

FIGURE 5: Equilibrium suggested from Millipore assays. The two complexes are thermodynamically highly favored over a mixture of separate components, but the relative amounts of each complex are determined by the concentrations of GTP, GDP, and of aminoacyl-tRNA (Gordon, 1963).

amino acid charged tRNA were necessary to displace the GDP. Approximately 70% of the GDP could be displaced in this way, indicating that, in the presence of aminoacyl-tRNA, GTP competes more effectively with GDP for binding to T factor, with formation of the T–GTP–aminoacyl-tRNA complex previously characterized (Gordon, 1967). Thus, the addition of aminoacyl-tRNA allows the selectivity for GDP binding to be reversed.

Discussion

The experiments described show that T factor from *E. coli* will bind GDP strongly and with high selectivity, even in the presence of a large excess of GTP. For example, from Figure 2, when GTP and GDP are present in the ratio 9:1, 90% of the nucleotide bound is GDP and 10% GTP. Ravel *et al.* (1968), using the Millipore assay, also found preferential binding of the tritium label of GTP compared with GTP labeled in the terminal phosphate, but they attributed this to hydrolysis. When aminoacyl-tRNA was added, the equilibrium moved in favor of GTP binding, and nonradioactive GTP was able to compete effectively with the [^{3}H]GDP (Figure 4 above). Earlier data showing that the addition of aminoacyl-tRNA caused the disappearance of the bound GTP in the Millipore assay (Gordon, 1968; Ravel *et al.*, 1968; Ertel *et al.*, 1968a) must now be reinterpreted. In our setup, the radioactivity bound was actually the trace of GDP preexisting in the [^{3}H]GTP; addition of aminoacyl-tRNA not only resulted in the formation of a ternary complex that was Millipore filtrable, but also reversed the preference for GDP. The present finding of only low levels of T–GTP suggests that this complex is much more labile than T–GDP. The equilibria postulated on the basis of our findings are summarized in Figure 5.

The data presented in this paper were all based on the Millipore assay, which measures the radioactivity bound to material adsorbable to the nitrocellulose membrane. Earlier results (Gordon, 1967) showed that GTP, labeled with either ^{3}H or ^{32}P, bound to high molecular weight material in the excluded volume of Sephadex columns, but only in the presence of aminoacyl-tRNA. The reason why the T–GDP complex described here did not appear in the earlier Sephadex analyses was attributed to its relative instability (Gordon, 1968). However, when the small amount of radioactivity that was excluded from Sephadex in the absence of aminoacyl-tRNA was analyzed, a preferential binding of GDP was found in that case too. The reason for the apparent differences in stability of the same complex obtained with the two assays is not clear. It may be that the Millipore itself has some stabilizing effect. This is consistent with the fact that the groups that are involved in the interaction with tRNA are also involved in the binding to the Millipore (concluded from the reversal of the Millipore binding by aminoacyl-tRNA; Gordon, 1968).

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