# *STEPWISE SYNTHESIS OF A TRIPEPTIDE**

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The question of the mode of action of the *E. coli* supernatant factors T[1, 2] ($T_u$ and $T_s$)[3] and G[1, 2, 4] has remained open until recently. It is now clear that the T factor, together with GTP, participates in the attachment of aminoacyl-tRNA's to ribosomes.[5–7] After the binding reaction, peptide bond formation ensues if the peptidyl site carries peptidyl-tRNA,[8] or an aminoacyl-tRNA with a free[6, 9] or a blocked[6, 7] $\alpha$-amino group on the amino acid. Polymerization ceases at this stage and continues only upon addition of G factor.

It has been proposed by Nishizuka and Lipmann[10] that GTP and the G factor[11] are involved in messenger RNA movement and, simultaneously, in translocation[12] of the newly synthesized peptidyl-tRNA from the aminoacyl to the peptidyl site. The completion of this process opens the aminoacyl site for entry of the next aminoacyl-tRNA. This possibility has been examined by various laboratories. Using a mammalian system, Skogerson and Moldave[8] have shown that aminoacyl transferase II (analogous to G factor in mammalian systems) and GTP lead to the transposition of peptidyl-tRNA from the aminoacyl to the peptidyl site. The work of Erbe and Leder[7] indicates that whereas only T and GTP are required to form F-Met-Phe-tRNA with AUG ($U_6$) as template, the translation of the third triplet codon depends on G.

In this paper we elaborate further on the T-dependent binding of aminoacyl-tRNA to ribosomes and show (in agreement with Skogerson and Moldave[8]) that if GTP is replaced by GMP-PCP, the aminoacyl-tRNA is bound, but subsequent peptide bond formation is prevented with prebound *N*-acetylPhe-tRNA. The aminoacyl-tRNA bound in this way does not interfere with the puromycin release of the prebound *N*-acetylPhe.

We also describe experiments which concern the possible role of the G factor in polymerization. By stepwise addition of aminoacyl-tRNA's to ribosomes, we have synthesized *N*-acetyltriPhe-tRNA, and demonstrate that after one peptide has been formed, an incubation period with G factor and GTP is required before the peptide chain can be lengthened by another amino acid. A similar approach to this problem was undertaken by Schweet and his collaborators[13, 14] in a reticulocyte cell-free system.

*Methods.—Preparation of ribosomes and supernatant factors:* *E. coli* B cells were harvested in mid-log phase[2] and were used to prepare ribosomes as reported previously.[6] The supernatant factors T ($T_u$ and $T_s$) and G were isolated from *Ps. fluorescens*, and the T fraction was separated into a $T_s$ peak, a $T_uT_s$ peak, and a peak enriched in $T_u$ by chromatography on a DEAE-cellulose column as described by Lucas-Lenard and Lipmann.[15]

*Acetylation of phenylalanyl-tRNA:* $C^{14}$-Phe-tRNA was acetylated as described by Haenni and Chapeville.[16]

*Binding of N-acetyl-$C^{14}$-Phe-tRNA and of $H^3$-Phe-tRNA to ribosomes; analysis of the products:* The reaction mixture (incubation I) contained, in a total volume of 1 ml: 50 $\mu$moles of Tris-HCl, pH 7.4, 10 $\mu$moles of magnesium acetate, 160 $\mu$moles of $NH_4Cl$, 10 $\mu$moles of DTT, 40 $\mu$g of poly U, and approximately 9.6 $A_{260}$ units of ribosomes. To this

mixture were added 120 $\mu$g of *N*-acetyl-$C^{14}$-Phe-tRNA containing 80 $\mu\mu$moles of $C^{14}$-Phe. After incubation for 20 min at 30°, the samples were cooled to 0° and, where indicated, supplemented with 1.6 $\mu$g of T, 200 m$\mu$moles of GTP or 340 m$\mu$moles of GMP-PCP, and 120 $\mu$g of $H^3$-Phe-tRNA containing 72 $\mu\mu$moles of $H^3$-Phe. The mixtures were incubated for 10 min at 0°.

In some cases, as indicated in the legends to the tables, the reaction was stopped at this point by the addition of a large volume of a buffer mixture containing 0.01 *M* Tris-HCl, pH 7.4, 0.01 *M* magnesium acetate, and 0.16 *M* $NH_4Cl$. The samples were immediately filtered, as described by Nirenberg and Leder,[17] and counted.

In other experiments, the samples were layered over 1 ml of 10% sucrose containing 0.01 *M* Tris-HCl, pH 7.4, 0.01 *M* magnesium acetate, 0.16 *M* $NH_4Cl$, and 0.001 *M* DTT, and were centrifuged for 1 hr at 150,000 × *g* (centrifugation I). The products bound to the ribosomal pellets through tRNA were freed by alkaline hydrolysis and analyzed by paper electrophoresis with a modification of the method of Lucas-Lenard and Haenni;[6] the electrophoresis was carried out at 32 v/cm for 4 hr.

*Puromycin release experiments:* The ribosomes recovered after centrifugation I and carrying double-labeled *N*-acetyldiPhe-tRNA were suspended in 0.01 *M* Tris-HCl, pH 7.4, and 0.01 *M* magnesium acetate (buffer A), and were used for further assays. They were incubated (incubation II) for 5 min at 30° in a 1-ml mixture containing 50 $\mu$moles of Tris-HCl, pH 7.4, 10 $\mu$moles of magnesium acetate, 160 $\mu$moles of $NH_4Cl$, 10 $\mu$moles of DTT, and the additions described in Tables 3 and 4. Puromycin (0.8 $\mu$mole) was then added and the incubation mixtures were maintained for two more minutes at 30°. The reaction was stopped with sodium acetate, pH 5, and the puromycin products were extracted by ethyl acetate as described by Leder and Bursztyn.[18]

*Synthesis of N-acetyltriPhe-tRNA:* Three incubations, each one followed by a centrifugation to recover the ribosomes carrying the labeled material, were used to synthesize *N*-acetyltriPhe-tRNA. Incubation I was carried out as indicated above. The ribosomes resuspended after centrifugation 1 were incubated for 5 min at 30° in the conditions described for incubation II and Table 5, except that puromycin was omitted. The samples were cooled, layered over sucrose solution, and spun (centrifugation II) as described above for centrifugation I. These ribosomes were shown to be free of G and GTP by polyphenylalanine polymerization.[3]

The ribosomal pellets were suspended in buffer A and incubated (incubation III) for 10 min at 0° in a 1-ml mixture containing 50 $\mu$moles of Tris-HCl, pH 7.4, 10 $\mu$moles of magnesium acetate, 160 $\mu$moles of $NH_4Cl$, 10 $\mu$moles of DTT, 1.6 $\mu$g of T, 200 m$\mu$moles of GTP, and 120 $\mu$g of $H^3$-Phe-tRNA. The samples were again layered over sucrose solution and spun (centrifugation III) as described above, thereby eliminating the excess $H^3$-Phe-tRNA. The ribosomal pellets were analyzed for bound products by paper electrophoresis as indicated above.

*Materials.*—Fusidic acid was obtained through the courtesy of Dr. W. O. Godtfredsen (Leo Pharmaceutical Products, Copenhagen, Denmark), and chlortetracycline (aureomycin) was supplied by Lederle. *N*-acetylPhe, *N*-acetyldiPhe, Phe, diPhe, and triPhe standards were purchased from Cyclo Chemical Corp. *N*-acetyltriPhe was prepared from triPhe as reported by Greenstein and Winitz.[19] GMP-PCP was obtained from Miles Laboratories, Inc.

*Results.—Requirement for both $T_s$ and $T_u$ in Phe-tRNA binding to ribosomes:* In our previous report,[6] we described the GTP- and T-dependent binding of Phe-tRNA to ribosomes and showed that GTP and $T_s$ alone did not promote this reaction. $T_s$ and $T_u$ were isolated as described from *Ps. fluorescens*.[3] Table 1 demonstrates that both are required for optimal binding of Phe-tRNA to the ribosomes. The slight effect observed by $T_u$ was due to some contamination of this fraction with $T_s$. Both $T_s$ and $T_u$ were also found to be needed for Millipore binding of $H^3$-GTP. These results agree with those of others,[20, 21] who observed that both $T_s$ and $T_u$ from *E. coli* were required for these reactions.

TABLE 1. *Requirement for both $T_s$ and $T_u$ to bind $H^3$-Phe-tRNA to ribosomes carrying prebound N-acetyl-$C^{14}$-Phe-tRNA.*

| Additions | $H^3$-Phe-tRNA bound ($\mu\mu$moles) |
|---|---|
| None | 2.8 |
| $T_s$ + GTP | 2.8 |
| $T_u$ + GTP | 5.2 |
| $T_s$ + $T_u$ + GTP | 11.6 |

The experimental conditions are described in *Methods*. Where indicated, 80 $\mu$g of $T_s$ and 40 $\mu$g of $T_u$ were added in place of combined T per milliliter incubation mixture. The Millipore filter technique[17] was used.

*Effect of GMP-PCP and GTP on T-directed binding of Phe-tRNA to ribosomes carrying N-acetylPhe-tRNA and on peptide bond formation:* Since GTP is a prerequisite for the attachment of aminoacyl-tRNA to ribosomes leading to dipeptide formation, we investigated the effect of GMP-PCP on this reaction. In accordance with the results of others,[20] we observed (Table 2) that GMP-PCP substitutes for GTP in promoting Phe-tRNA binding; both the rate and extent of binding are the same. As shown further in Table 2, however, the binding reaction with GMP-PCP does not lead to dipeptide formation. This is in contrast to the large stimulation of *N*-acetyldiPhe-tRNA synthesis observed with GTP.

Both ribosomal subunits are necessary for the binding reaction when either GMP-PCP or GTP is used. Furthermore, chlortetracycline inhibits this reaction, whichever nucleotide is present.

*Puromycin release of N-acetyldiPhe:* Our previous results,[6] as well as the findings reported here, show that in the presence of T and GTP polymerization stops at the dipeptide stage. Since the puromycin release of peptides is considered as a model of peptide bond formation, the requirements of this reaction were examined, using ribosomes carrying *N*-acetyldiPhe-tRNA. Table 3 shows that in the presence of puromycin alone there was some release of *N*-acetyldiPhe. This release could not be lowered by further washing of the ribosomes. However, when G and GTP as well as puromycin were added, all of the bound *N*-acetyldiPhe was released. Fusidic acid, an antibiotic known to inhibit the G factor and ribosome-dependent GTPase,[22] also blocked the G + GTP-dependent puromycin-release reaction. It did not decrease the background of release in the absence of added G and GTP. If this release is caused by G contamination on the ribosomes, then this factor must be protected from the action of fusidic acid while on the ribosomes.

TABLE 2. *Effect of GMP-PCP on T-directed binding of $H^3$-Phe-tRNA to ribosomes containing prebound N-acetyl-$C^{14}$-Phe-tRNA.*

| Additions | *N*-acetyl-Phe-tRNA ($C^{14}$) ($\mu\mu$moles) | *N*-acetyl-diPhe-tRNA ($C^{14}$ or $H^3$) ($\mu\mu$moles) | Phe-tRNA ($H^3$) ($\mu\mu$moles) |
|---|---|---|---|
| None | 7.6 | 0.4 | 0.1 |
| T + GTP | 3.0 | 4.5 | 2.8 |
| T + GMP-PCP | 7.0 | 0.5 | 4.8 |

The conditions of this experiment are described in *Methods*. The products were analyzed by paper electrophoresis.

TABLE 3. *Puromycin release of N-acetyldiPhe.*

| Additions | *N*-acetyldiPhe-puro released ($C^{14}$ or $H^3$) ($\mu\mu$moles) |
|---|---|
| Puro | 1.8 |
| G + GTP + puro | 5.4 |
| G + GTP + fusidic acid + puro | 2.0 |
| G + GTP + GMP-PCP + puro | 4.1 |
| G + puro | 2.0 |
| GTP + puro | 1.6 |

The experimental procedure is outlined in *Methods*. Ribosomes carrying 5.4 $\mu\mu$moles of *N*-acetyl, $C^{14}$-Phe-$H^3$-tRNA were used per milliliter incubation mixture, and, where indicated, 20 $\mu$g of G-200 m$\mu$moles of GTP, 340 m$\mu$moles of GMP-PCP, and 0.6 $\mu$mole of fusidic acid were added.

GMP-PCP was also observed to interfere with the G + GTP-stimulated release reaction. Neither G nor GTP was effective alone. Although not shown in the table, GDP or GMP-PCP could not replace GTP.

In Table 3, only the release of *N*-acetyldiPhe is recorded. However, as suggested by Table 2, some ribosomes may have carried only *N*-acetylPhe-tRNA or Phe-tRNA. Puromycin reacted with *N*-acetylPhe, but hardly at all (less than 5%) with the Phe-tRNA, even in the presence of G and GTP, as measured by ethyl acetate extraction at pH 8,[18] or by the Millipore binding assay.[17]

*Puromycin release of N-acetylPhe; noninterference by the presence of Phe-tRNA bound to ribosomes with T and GMP-PCP:* It is shown in Table 2 that GMP-PCP can replace GTP in the binding of the Phe-tRNA to ribosomes carrying *N*-acetylPhe-tRNA, but that no *N*-acetyldiPhe-tRNA is formed. We then investigated whether the bound Phe-tRNA would interfere with the puromycin release of the prebound *N*-acetylPhe-tRNA. Experiment 1 in Table 4 demonstrates that the presence of the Phe-tRNA on the ribosomes did not block the ability of puromycin to release the *N*-acetylPhe. The level of *N*-acetylPhe released was similar to that observed in the absence of any added Phe-tRNA (expt. 2). Experiment 3 shows what happens when GTP is used to bind Phe-tRNA. As expected, most of the *N*-acetylPhe was recovered as *N*-acetyldiPhe, which was released by puromycin largely only in the presence of G and GTP (see also Table 3). Saturating levels of *N*-acetylPhe- and Phe-tRNA were used.

TABLE 4. *Binding of $H^3$-Phe-tRNA to ribosomes carrying N-acetyl-$C^{14}$-Phe-tRNA; puromycin release of reaction products.*

| Conditions of incubation I | Additions in incubation II | *N*-acetylPhe-puro released ($C^{14}$) ($\mu\mu$moles) | *N*-acetyldiPhe-puro released ($C^{14}$ or $H^3$) ($\mu\mu$moles) |
|---|---|---|---|
| 1. Phe-tRNA bound with T + GMP-PCP | Puro | 12.4 | 0 |
| | G + GTP + puro | 12.4 | 1.2 |
| 2. No Phe-tRNA | Puro | 11.6 | — |
| | G + GTP + puro | 12.6 | — |
| 3. Phe-tRNA bound with T + GTP | Puro | 2.2 | 1.9 |
| | G + GTP + puro | 2.8 | 7.3 |

After incubation I, the ribosomes carried, per milliliter incubation mixture: (1) 13.6 $\mu\mu$moles of *N*-acetylPhe-tRNA, 1.2 $\mu\mu$mole of *N*-acetyldiPhe-tRNA, and 18.4 $\mu\mu$moles of Phe-tRNA; (2) 14.8 $\mu\mu$moles of *N*-acetylPhe-tRNA; and (3) 7.5 $\mu\mu$moles of *N*-acetylPhe-tRNA, 7.3 $\mu\mu$moles of *N*-acetyldiPhe-tRNA, and 11.2 $\mu\mu$moles of Phe-tRNA. Where indicated, 20 $\mu$g of G and 200 m$\mu$moles of GTP were added in incubation II (see *Methods*).

To see if Phe-tRNA was still present on the ribosomes after treatment with puromycin in experiment 1, the incubation mixture was centrifuged and the products bound to the ribosomes were measured by the Millipore binding assay.[17] Over 70 per cent of the Phe-tRNA was recovered on the ribosomes.

*Formation of N-acetyltriPhe-tRNA:* If, as shown by the puromycin release of *N*-acetyldiPhe, the formation of a second peptide bond requires the presence of G and GTP, then adding Phe-tRNA to *N*-acetyldiPhe-tRNA to form *N*-acetyltriPhe-tRNA would be expected to have the same requirements. Ribosomes carrying *N*-acetyldiPhe-tRNA were incubated under various conditions as indicated in *Methods* and in Table 5. The ribosomes recovered after centrifugation II and now free of detectable G and GTP were again incubated with T, GTP, and $H^3$-Phe-tRNA and spun a third time (centrifugation III). The ribosomal products were analyzed by paper electrophoresis.

Experiment 1 of Table 5 shows that there was an eightfold increase in the amount of *N*-acetyltriPhe-tRNA formed when the ribosomes containing *N*-acetyldiPhe-tRNA were incubated with G and GTP. Though not shown in the table, this was accompanied by a decrease in the amount of *N*-acetyldiPhe-tRNA. Fusidic acid greatly inhibited the formation of *N*-acetyltriPhe-tRNA.

Experiment 2 of Table 5 demonstrates that neither G nor GTP alone was effective. As with the puromycin reaction, GMP-PCP could not substitute for GTP. The recovery of products in experiment 2 was lower than that in experiment 1, because of aging of the ribosome preparation, whereupon considerable loss of the bound counts occurred during centrifugations II and III. The supernatants recovered after these centrifugations were analyzed by paper electrophoresis; the losses were not specific for one type of product. The recovery of experiment 1 was 90 per cent.

*Discussion.*—The use of *N*-acetylPhe-tRNA as initiator in poly U-directed polyphenylalanine synthesis has proved advantageous for separately studying the roles of the supernatant factors T and G. With *N*-acetylPhe-tRNA bound to the peptidyl site, we have shown that T ($T_s$ and $T_u$) and GTP cause the attach-

TABLE 5. *Formation of N-acetyltriPhe-tRNA by addition of Phe-tRNA to ribosomes carrying N-acetyldiPhe-tRNA.*

| Treatment of ribosomes before incubation with Phe-tRNA | *N*-acetyltriPhe-tRNA formed ($\mu\mu$moles) ($C^{14}$) |
|---|---|
| Experiment 1 | |
| None | 0.5 |
| G + GTP | 3.8 |
| G + GTP + fusidic acid | 1.3 |
| Experiment 2 | |
| None | 0.3 |
| G + GTP | 1.1 |
| G | 0.2 |
| GTP | 0.2 |

The conditions of this experiment are described in *Methods*. Where indicated, 20 $\mu$g of G, 200 m$\mu$moles of GTP, and 0.6 $\mu$mole of fusidic acid were added per milliliter incubation mixture to ribosomes carrying *N*-acetyl-$C^{14}$-Phe-$H^3$-Phe-tRNA. After reisolation, the ribosomes were incubated with T, GTP, and $H^3$-Phe-tRNA under the usual conditions. Only the amount of *N*-acetyltriPhe-tRNA formed is reported.

ment of aminoacyl-tRNA to the aminoacyl site. *N*-acetyldiPhe-tRNA synthesis succeeds this binding reaction, since the peptidyl transferase is part of the 50*S* ribosomal subunit.[23]

In this paper, we have described some additional studies on the binding of aminoacyl-tRNA to ribosomes. We find that when GTP is replaced by GMP-PCP, Phe-tRNA binding occurs nearly as well as with GTP; however, *N*-acetyldiPhe-tRNA formation is prevented. In spite of the presence of Phe-tRNA on these ribosomes, puromycin, an analogue of the aminoacyl adenosine terminus of aminoacyl-tRNA, can still react, releasing all of the bound *N*-acetylPhe. It is possible that GTP induces a crucial alignment of aminoacyl-tRNA on the ribosomes such that the aminoacyl adenosine end is available for peptide bond formation on the 50*S* subunit. This critical attachment does not occur when GMP-PCP replaces GTP. These findings indicate a difference between mere binding and binding leading to the formation of a peptide bond. A parallel can be drawn between these results and those obtained in chain initiation by Takeda and Webster,[24] and others;[25, 26] GMP-PCP substitutes for GTP in the $F_1 + F_2$-promoted binding of F-Met-tRNA but F-Met-puro is formed only when GTP has been used. It remains to be determined if the need for GTP in these reactions means a split of GTP. Peptide formation *per se* probably does not require GTP, as shown by various authors.[23, 27, 28]

We also show that after the synthesis of *N*-acetyldiPhe-tRNA no further peptide bonds can be formed, unless the ribosomes carrying the dipeptidyl-tRNA are first incubated with G and GTP. Then, after removal of G and GTP by centrifugation, subsequent treatment either with Phe-tRNA, T, and GTP or with puromycin results in *N*-acetyltriPhe-tRNA or *N*-acetyldiPhe-puro synthesis, respectively. The effect of G and GTP might be explained in terms of the translocation model of protein synthesis.

Earlier experiments by Nishizuka and Lipmann[2] have indicated approximate equivalence between peptide bond formation and phosphate liberation from GTP, as determined by poly U-directed polyphenylalanine synthesis. This hydrolysis was attributed to the concerted action of ribosomes, G factor, and GTP in translocation. The finding that GTP is required for a number of steps in polymerization makes it desirable to reassess the over-all liberation of phosphate related to peptide synthesis.

*Summary.*—Both $T_s$ and $T_u$, the subfractions of the T factor, and GTP are required to bind Phe-tRNA to ribosomes, and *N*-acetyldiPhe-tRNA is formed if the ribosomes carry *N*-acetylPhe-tRNA. GMP-PCP substitutes efficiently for GTP in the binding reaction, but no dipeptide is formed. However, Phe-tRNA bound with GMP-PCP does not interfere with the puromycin release of *N*-acetylPhe, suggesting that GMP-PCP prevents correct binding of the aminoacyl end of the Phe-tRNA on the 50*S* subparticle.

If ribosomes carrying *N*-acetyldiPhe-tRNA are first incubated with G and GTP, puromycin or an additional Phe-tRNA can subsequently be bound to the ribosomes, resulting in *N*-acetyldiPhe-puro or *N*-acetyltriPhe-tRNA synthesis.

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Abbreviations: Phe, phenylalanine; diPhe, diphenylalanine; triPhe, triphenylalanine; *N*-acetylPhe, *N*-acetylphenylalanine; *N*-acetyldiPhe, *N*-acetyldiphenylalanine; *N*-acetyltriPhe, *N*-acetyltriphenylalanine; Phe-tRNA, phenylalanyl-tRNA; *N*-acetylPhe-tRNA, *N*-acetylphenylalanyl-tRNA; *N*-acetyldiPhe-tRNA, *N*-acetyldiphenylalanyl-tRNA; *N*-acetyltriPhe-tRNA, *N*-acetyltriphenylalanyl-tRNA; F-Met-Phe-tRNA, formylmethionylphenylalanyl-tRNA; puro, puromycin; *N*-acetylPhe-puro, *N*-acetylphenylalanylpuromycin; *N*-acetyldiPhe-puro, *N*-acetyldiphenylalanylpuromycin; F-Met-puro, formylmethionylpuromycin; DTT, dithiothreitol; GMP-PCP, 5′-guanylyl methylenediphosphonate; Tris, tris (hydroxymethyl)aminomethane; DEAE-cellulose, *O*-(diethylaminoethyl)cellulose; GTP, guanosine 5′-triphosphate; GTPase, guanosine triphosphatase.

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