Peptide Chain Elongation. Role of the S_1 Factor in the Pathway from S_3–Guanosine Diphosphate Complex to Aminoacyl Transfer Ribonucleic Acid–S_3–Guanosine Triphosphate Complex*

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ABSTRACT: We have been studying the mechanism of peptide chain elongation in a cell-free system in which polyuridylic acid directs the conversion of Phe-tRNA to Phe_n-tRNA. The system includes ribosomes from *Escherichia coli* and purified peptide chain elongation factors S_1, S_2, and S_3 from *Bacillus stearothermophilus*. (These factors correspond to the following factors from *E. coli* and *Pseudomonas fluorescens:* S_1 to T_s and TI_s, S_2 to G and TII, and S_3 to T_u and TI_u.) The addition of each AA-tRNA to the growing peptide chain is a multistep process which can be divided into three phases: (1) AA-tRNA binding, (2) peptide-bond formation, and (3) translocation. The following events were known to occur in phase 1: (i) AA-tRNA is bound to the A site of the ribosome–mRNA complex (carrying peptidyl-tRNA in the P site) in the form of an AA-tRNA–S_3–GTP complex; (ii) after binding, but before peptide-bond formation, the GTP in the complex is cleaved, and an S_3–GDP complex and inorganic phosphate are released from the ribosome; (iii) S_1 promotes the re-formation of the AA-tRNA–S_3–GTP complex from S_3–GDP complex, GTP, and AA-tRNA. In this communication we present data on the intermediate steps in iii. S_1 causes release of GDP from the S_3–GDP complex forming an S_1–S_3 complex. This reacts with GTP, S_1 is released, and an S_3–GTP complex is formed. Finally, the S_3–GTP complex binds AA-tRNA thus forming AA-tRNA–S_3–GTP complex.

Most of our studies on peptide chain elongation have been performed in a simple model system in which poly(U) promotes the conversion of Phe-tRNA[1] to Phe_n-tRNA (Lipmann, 1969; Lengyel and Söll, 1969). This system includes GTP, salts, ribosomes from *Escherichia coli*, and purified peptide chain elongation factors S_1, S_2, and S_3 from *Bacillus stearothermophilus* (Skoultchi *et al.*, 1968). These factors are analogous to those from *E. coli* and *Pseudomonas fluorescens*. S_1 corresponds to T_s and TI_s, S_2 to G and TII, and S_3 to T_u and TI_u (Lucas-Lenard and Lipmann, 1966; Ravel *et al.*, 1970). Subsequently, when discussing a factor from these two microorganisms we shall note in parentheses the designation of the corresponding factor from *B. st.*

Studies with the above system contributed to the elucidation of the following scheme of peptide chain elongation: the addition of each aminoacyl residue to the growing peptidyl-tRNA chain is a cyclic process (peptide chain elongation cycle). In the first phase of the cycle (*AA-tRNA binding*) an AA-tRNA–S_3–GTP complex is bound to the AA-tRNA binding site (or A site) of the ribosome–mRNA complex which has peptidyl-tRNA bound at the peptidyl-tRNA binding site (or P site) (Skoultchi *et al.*, 1969, 1970; Lucas-Lenard *et al.*, 1969; Brot *et al.*, 1970; see also Ravel, 1967; Gordon, 1968; Ertel *et al.*, 1968; Shorey *et al.*, 1969; Lockwood *et al.*, 1971). In the second phase (*peptide-bond formation*) the GTP from the ribosome-bound AA-tRNA–S_3–GTP complex is cleaved, S_3–GDP complex (also designated as complex III) and P_i are released from the ribosome (Shorey *et al.*, 1969; Haenni *et al.*, 1968; Ono *et al.*, 1969a,b; Gordon, 1969; Skoultchi *et al.*, 1970; Waterson *et al.*, 1970; Lockwood *et al.*, 1971), and the peptidyl residue of the peptidyl-tRNA is released from its linkage to tRNA and forms a peptide bond with the α amino group of the AA-tRNA in the A site (Monro *et al.*, 1969; Skoultchi *et al.*, 1969). In the third phase (*translocation*) S_2 bound to the ribosome triggers the cleavage of further GTP, the discharged tRNA is released from the P site, the peptidyl-tRNA (which has just been extended by one aminoacyl residue) is shifted from the A site to the P site (Traut and Monro, 1964; Brot *et al.*, 1968; Haenni and Lucas-Lenard, 1968; Kuriki and Kaji, 1968; Pestka, 1968; Erbe *et al.*, 1969; Lucas-Lenard and Haenni, 1969), and the ribosome moves the length of one codon along the mRNA in the 5′ to 3′ direction (Gupta *et al.*, 1971). Finally, S_1 promotes the re-formation of the AA-tRNA–S_3–GTP complex from S_3–GDP complex (complex III), GTP, and AA-tRNA (Weissbach *et al.*, 1970a; Waterson *et al.*, 1970). This finishes the process, and the stage is set for attaching another AA-tRNA to the peptide chain by repeating the cycle.

In this communication we present studies on the intermediate steps in the pathway from S_3–GDP complex (complex III) to AA-tRNA–S_3–GTP complex and the role of S_1 in the process. The results obtained confirm and extend those obtained by studies with elongation factors from *E. coli* (Weissbach *et al.*, 1970a,b, 1971).

Experimental Section

Materials

PEP and pyruvate kinase were obtained from Calbiochem;

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[1] Abbreviations used are: *B. st.*, *Bacillus stearothermophilus;* Phe-tRNA, phenylalanyl-tRNA; Phe_n-tRNA, polyphenylalanyl-tRNA; AA-tRNA, aminoacyl-tRNA; DTT, dithiothreitol; PEP, phosphoenolpyruvate trisodium salt.

E. coli B tRNA, L-[^{14}C]phenylalanine (specific activity 455 Ci/mole), [^{3}H]GDP (specific activity 1770 Ci/mole), and [^{3}H]-GTP (specific activity 1160 Ci/mole) from Schwarz–Mann. [γ-^{32}P]GTP (specific activity 2700 Ci/mole) prepared according to the procedure of Glynn and Chappell (1964) for preparing [γ-^{32}P]ATP was a gift from Dr. Y. Ono.

Methods

Components of the Cell-Free System. All the reaction mixtures contained standard buffer (0.01 M Tris-HCl (pH 7.4)–0.16 M NH_4Cl–0.01 M magnesium acetate) as well as 0.01 M DTT. Standard buffer supplemented with 0.001 M DTT was used, unless otherwise specified, for equilibrating and eluting Sephadex columns at 4° and for preparing sucrose gradients. [^{14}C]Phe-tRNA (specific activity 120 Ci/mole) and thoroughly washed ribosomes both from *E. coli* were prepared according to published procedures (Skoultchi *et al.*, 1968).

Purification of S_1, S_2, and S_3 from B. st. S_1 was purified by a procedure modified from that of Skoultchi *et al.* (1968). The DEAE-Sephadex A-50 column was eluted with a linear gradient of KCl (0.15–0.55 M) in 0.01 M potassium cacodylate (pH 6.4)–0.001 M DTT. S_1 activity appeared in the eluate when the concentration of KCl reached 0.2 M. The concentration of S_1 activity remained the same till the KCl concentration reached 0.35 M. By this time 50% of the total S_1 activity was eluted (leading S_1). The remaining S_1 activity was eluted as a peak centered at 0.37 M KCl (peak S_1). The fractions containing leading S_1 were pooled separately from those containing peak S_1. The leading S_1 was further purified by repeating the chromatography on DEAE-Sephadex A-50 under the same conditions as previously. The S_1 activity emerged this time as a peak at about 0.2 M KCl. The fractions in this peak were pooled. The S_1 in these moved as a single band on polyacrylamide gel electrophoresis in 8 M urea at both pH 4.5 (Reisfeld *et al.*, 1962) and 8.9 (Davis, 1964). (It was free of S_3 activity as examined by the polymerization assay.) This S_1 preparation was used in the experiments described in this communication. The peak S_1 was contaminated with S_2 activity. It was further purified by (i) chromatography on a DEAE-Sephadex A-50 column equilibrated with 0.15 M KCl in a 0.01 M Tris-HCl (pH 7.4)–0.001 M DTT solution. The column was eluted with a linear concentration gradient of KCl (0.15–0.55 M) in the same solution. (ii) The active fraction was further purified by chromatography on a hydroxylapatite column. This was eluted with a linear gradient of potassium phosphate (pH 7.4, 0.01–0.2 M). The S_1 activity was eluted as a peak at 0.04 M potassium phosphate concentration. This S_1 preparation was estimated to be 80% pure, free of S_2 activity, and containing less than 5% S_3 activity. This enzyme preparation was used in obtaining an antiserum against S_1 (Waterson *et al.*, 1970).

S_2 (purified 70-fold) and S_3 (purified 100-fold) were obtained as described earlier (Skoultchi *et al.*, 1968). The purified S_3 preparation was apparently homogeneous, *i.e.*, moved as a single band on electrophoresis in 8 M urea on polyacrylamide gel at both pH 4.5 and 8.9. S_3 was crystallized recently in the presence of GDP in the form of an S_3–GDP complex (M. L. Sopori, unpublished data)

Molecular Weights and Sedimentation Coefficients of the Elongation Factors. The molecular weights of the elongation factors were examined by electrophoresis in sodium dodecyl sulfate on polyacrylamide gels, according to the procedure of Shapiro *et al.* (1967). The molecular weight of S_1 was found to be about 37,000, that of S_2 about 85,000, and that of S_3 about 51,000 (Waterson, 1971).

The *s* values of the factors were examined by centrifugation through sucrose gradient (Martin and Ames, 1961), alkaline phosphatase and cytochrome *c* serving as standards. The *s* value of S_1 was found to be 2.6, that of S_2 4.8, and that of S_3 3.1. The *s* value of S_3 was also determined by sedimentation velocity measurement in the analytical ultracentrifuge. For this purpose S_3 (approximately 400 μg/ml) was dialyzed overnight in the cold against standard buffer with 0.001 M DTT. The resulting solution was supplemented with 3×10^{-5} M GDP to form S_3–GDP complex, incubated at 30° for 10 min, and cooled to 0°. The sedimentation coefficient was determined according to Schachman (1957) in a Beckman Model E ultracentrifuge, equipped with uv optics and an AND rotor. A double-sector cell (with 12-mm optical pathway and sapphire windows) was filled with the above S_3–GDP solution in one sector, and the control cell was filled with 2×10^{-5} M GDP and 0.001 M DTT in standard buffer. The speed of the rotor was 52,640 rpm and its temperature was 8°. Using the uv scanning system, the movement of the boundary was recorded every 16 min. At least ten points were used for the plot of log *r* as a function of time; the *s* values observed on five runs were averaged and corrected to $s_{20,w}$; the mean value was 3.12 $s_{20,w}$, the standard error was 0.081 $s_{20,w}$.

Preparation of S_3 Free of Guanine Nucleotides. Purified Tu (S_3) contains bound GDP (Miller and Weissbach, 1970a). For studies on the GTP requirement of AA-tRNA binding to S_3 we prepared S_3 essentially free of guanine nucleotides according to a procedure modified from that of Shorey *et al.* (1969).

A [^{14}C]Phe-tRNA–S_3–[^{3}H]GTP complex was prepared by incubating [^{14}C]Phe-tRNA carrying 125 pmoles of phenylalanyl residues (specific activity 120 Ci/mole, 817 A_{260} units); S_1, 2.1 μg; S_3 (containing bound GDP), 15.6 μg; [^{3}H]GTP, 0.017 μmole (specific activity 1160 Ci/mole); pyruvate kinase, 23.3 μg; and PEP, 0.46 μmole, in a volume of 0.2 ml at 30° for 4 min. In order to dissociate the complex and to separate the S_3 and the Phe-tRNA from the guanine nucleotides the reaction mixture was filtered through a Sephadex G-25 column (volume 4.15 ml) which had been equilibrated with a buffer containing 0.04 M Tris-HCl (pH 7.4), 0.16 M NH_4Cl, 0.002 M DTT, and 0.0015 M EDTA. The excluded fraction (detected by assaying for ^{14}C counts in Phe-tRNA in the effluent) was isolated. It was verified that the excluded fraction contained S_3 and Phe-tRNA as well as a small amount of guanine nucleotides corresponding to less than 5% of the amount of S_3 in moles.

Polymerization Assay of S_1 and S_3 Activity. These were performed by supplementing the material to be tested with all components required for Phe_n-tRNA synthesis except the factor (*i.e.*, S_1 or S_3) to be quantified. The reaction mixture (0.12 ml) contained in addition to standard buffer S_2, 0.09 μg; ribosomes, 1.42 A_{260} units; [^{14}C]Phe-tRNA carrying 25 μmoles of phenylalanyl residues (specific activity 120 Ci/mole; 1.76 A_{260} units); GTP, 250 μmoles; DTT, 10 μmoles; and either S_1, 0.36 μg, or S_3, 0.62 μg. Incubation was at 30° for 10 min. Subsequently the amount of [^{14}C]phenylalanine incorporated into hot acid-insoluble material was determined (Skoultchi *et al.*, 1968).

Complex III and S_3–GDP Complex. Though the above two are apparently identical, we will distinguish them according to the way in which they are obtained. Thus, we will designate as complex III the product containing S_3 and bound GDP isolated from an incubation mixture in which Phe-tRNA–S_3–GTP complex was reacted with a poly(U)–ribosome complex. (For details of this procedure, see Waterson *et al.*, 1970.)

We will designate as S_3–GDP complex a product obtained by incubating purified S_3 (usually containing some bound GDP) with excess GDP and small amounts of S_1. The S_1 was only added because we needed S_3–[^{3}H]GDP complex, and wanted to make sure that the unlabeled GDP bound to S_3 will be equilibrated with the added [^{3}H]GDP. Both complex III and S_3–GDP complex were, if so indicated, freed of S_1 activity by treatment with anti-S_1 antiserum. (For details, see Waterson *et al.*, 1970, and the legends to figures and tables.)

Conversion of GDP (Contaminating the GTP Preparation) to GTP. GTP (unlabeled or labeled with ^{3}H or ^{32}P) at the concentration required for the experiment (between 2×10^{-5} and 0.001 M) in standard buffer was supplemented with 216 μg/ml of pyruvate kinase and 0.0037 M PEP and incubated at 30° for 15 min to convert any GDP in the preparation to GTP (Cooper and Gordon, 1969). Subsequently the reaction mixture was cooled to 0° and (unless otherwise stated) extracted by shaking with an equal volume of water saturated, redistilled phenol for 10 min, and centrifuged to separate the phases. The aqueous phase was extracted four times with three volumes of ether to remove phenol and the ether was removed by bubbling through nitrogen. In the case of [γ-^{32}P]GTP the final solution was filtered through a Millipore filter to remove insoluble impurities. The GTP preparation taken through this procedure is designated subsequently as pretreated GTP.

Millipore Assay for Complex III, S_3–GDP Complex, and S_3–GTP Complex. This assay is based on the observation that GDP or GTP (not bound to protein) is not retained by Millipore filters whereas GDP or GTP in complex with S_3 is (Allende *et al.*, 1967). The reaction mixture (usually in 0.1-ml volume) was diluted with 1 ml of ice-cold standard buffer and filtered through an (HA25) Millipore filter. The filter was washed three times with 3 ml of standard buffer, dried, and counted in a toluene-based scintillator. The efficiency of counting was 10% for ^{3}H and 75% for ^{32}P in both single- and double-label experiments.

Results

S_1 Promotes the Formation of S_3–GTP Complex from Complex III and GTP. COMPETITION BETWEEN GTP AND GDP FOR BINDING TO S_3. During studies on the formation of AA-tRNA–S_3–GTP complex from complex III, GTP, and AA-tRNA, and the role of S_1 in this pathway, formation of an S_3–GTP complex was observed (Waterson *et al.*, 1970). Such a complex is a probable intermediate in the above pathway (Shorey *et al.*, 1969). It could arise by an exchange of the GDP moiety of complex III with GTP. We tested therefore if S_1 catalyzes this exchange. (S_1 was found earlier to promote the exchange of free GDP with GDP in complex III (Waterson *et al.*, 1970) and in S_3–GDP complex (Weissbach *et al.*, 1970a; see also Kawakita *et al.*, 1971).)

The curves in Figure 1A reveal that S_1 does promote an exchange between free GTP (labeled with ^{32}P in the γ position) and the GDP moiety in complex III (labeled with ^{3}H). However only less than half of the GDP from complex III was exchanged for GTP, though GTP was present in the reaction mixture in a 200-fold excess over complex III. The incompleteness of the exchange, which may be due to competition between GTP and GDP for binding to S_3, is consistent with other observations indicating that S_3 has a higher affinity for GDP than for GTP (Cooper and Gordon, 1969; Miller and Weissbach, 1970a).

To overcome this competition and to show that all (or essentially all) of complex III can be transformed into S_3–GTP complex in the presence of S_1 and GTP, the experiment was repeated in the presence of PEP and pyruvate kinase. These reagents were added to *remove GDP released from complex III from competition by phosphorylating it to GTP* (Cooper and Gordon, 1969; Weissbach *et al.*, 1970a). The curves in Figure 1B reveal that in these conditions the exchange of guanine nucleotides is promoted by S_1 and reaches completion. Another possible explanation for the enhancement of the rate

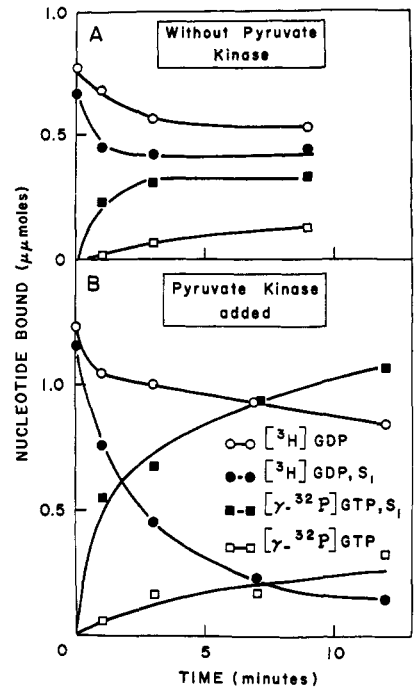

FIGURE 1: S_1 promotes the formation of S_3–GTP complex from complex III and GTP; competition between GTP and GDP for binding to S_3. Complex III, free of S_1 activity, was prepared according to the following procedure (for further details, see Waterson *et al.*, 1970). First, Phe-tRNA–S_3–[^{3}H]GTP complex was obtained by incubation of Phe-tRNA, S_1, S_3, and [^{3}H]GTP to form the complex, fractionation on a Sephadex G-25 column to remove [^{3}H]GTP not bound in the complex, and incubation with anti-S_1 antiserum to inactivate the S_1 present. The reaction mixture was then supplemented and incubated with a preformed poly(U)–ribosome complex to allow the binding of Phe-tRNA (from the Phe-tRNA–S_3–[^{3}H]-GTP complex) to the ribosome with the concomitant GTP cleavage and formation of complex III. Complex III was isolated by fractionating the reaction mixture on Sephadex G-150 and pooling the appropriate fractions. (A) Complex III (0.06 ml) containing 1.93 pmoles of [^{3}H]GDP (specific activity 1160 Ci/mole) at 0° was supplemented at zero time with S_1 (0.21 μg; if so indicated) and with pretreated [γ-^{32}P]GTP (390 pmoles; specific activity 276 Ci/mole), in a final volume of 0.085 ml. After incubation at 0° for the times shown the reaction mixtures were tested by the Millipore assay to determine the amount of complex III (^{3}H counts retained on the filter) and S_3–GTP complex (^{32}P counts retained on the filter) (see Methods). The amount of [^{32}P]GTP bound to the filter in the absence of complex III (0.12 pmole) was subtracted from each value. (B) This experiment was performed similarly to that in part A, except that the pyruvate kinase and PEP were present during the whole experiment in order to convert all free GDP to GTP. Complex III (0.07 ml) containing 2.32 pmoles of [^{3}H]GDP (specific activity 1160 Ci/mole) at 0° was supplemented at zero time with S_1 (0.21 μg; if so indicated) and with reaction mixture A (0.02 ml; containing [γ-^{32}P]GTP (500 pmoles; specific activity approximately 360 Ci/mole), PEP (74 nmoles), and pyruvate kinase (4.3 μg) that had been incubated previously at 30° for 15 min to convert all GDP in the solution to GTP. Incubation of the reaction mixture (final volume 0.09 ml) and the assay were performed as described for the experiment in part A. The amount of [γ-^{32}P]GTP bound to the filter in the absence of complex III (0.185 pmole) was subtracted from each value.

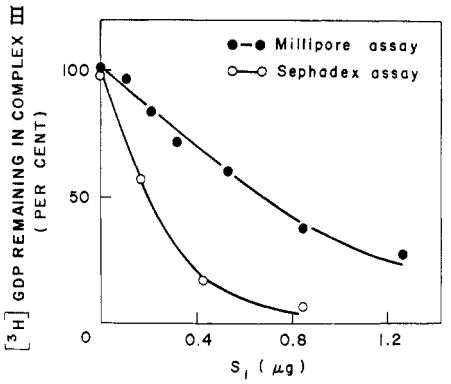

FIGURE 2: S_1 promotes the release of GDP from complex III. Millipore assay: different amounts of S_1 (as indicated) were added at 0° to aliquots of complex III (containing 1.56 pmoles of [^{3}H]GDP, specific activity 1160 Ci/mole) in a final volume of 0.1 ml at zero time. After incubation at 0° for 12 min the amount of S_3–GDP complex was determined by the Millipore assay (see Methods). In the absence of S_1 0.85 pmole of [^{3}H]GDP was retained on the filter. The results obtained with added S_1 are expressed as per cents of this amount. Sephadex assay: different amounts of S_1 (as indicated) were added at 0° to aliquots of complex III (containing 5.75 pmoles of [^{3}H]GDP, specific activity 1160 Ci/mole) in a final volume of 0.2 ml at zero time. After incubation at 0° for 10 min the reaction mixture was fractionated by gel filtration on a Sephadex G-25 column (6.5 ml) at 0°. To determine the amount of [^{3}H]GDP in the excluded volume (*i.e.*, in the form of S_3–GDP complex) 0.40-ml fractions were collected and 0.30-ml aliquots of each were counted in Bray's (1960) scintillation fluid. In the absence of S_1 1.65 pmoles of [^{3}H]-GDP was in the excluded volume. The results with added S_1 are expressed as per cents of this amount.

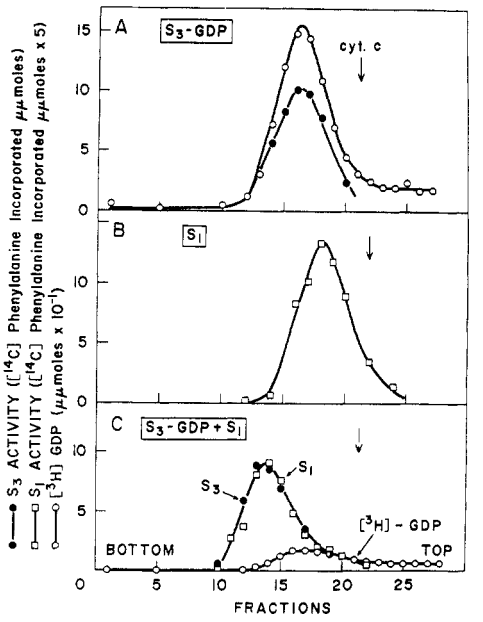

FIGURE 3: The reaction of S_3–GDP complex with S_1 results in the formation of an S_1–S_3 complex and the release of GDP. S_3 (7.8 μg) was supplemented with [^{3}H]GDP (800 pmoles; specific activity 1770 Ci/mole) and with either a small amount of S_1 (0.42 μg; sufficient to equilibrate the labeled GDP with the unlabeled GDP bound to S_3, Figure 3A) or with excess S_1 (8.6 μg; resulting in the binding of most of the S_3 in an S_1–S_3 complex and the release of about 80% of the GDP from its attachment to S_3, Figure 3C). After incubating each reaction mixture (0.1-ml final volume) at 30° for 10 min each was fractionated by gel filtration on a Sephadex G-25 column (volume 3 ml) to separate free [^{3}H]GDP from the macromolecular components. An aliquot of the pooled excluded fractions (0.2 ml) was mixed with a colored marker (cytochrome *c*, 0.6 mg) and layered over a sucrose gradient (4.8 ml, 5–20%). S_1 (4.2 μg; in 0.2 ml) was also mixed with the cytochrome *c* marker and layered on top of a third sucrose gradient (Figure 3B). After centrifugation (at 46,500 rpm in a Beckman SW50.1 rotor at 4° for 16 hr) the gradient was collected in 27 to 28 fractions. The fractions in A were assayed for S_3, [^{3}H]GDP, and cytochrome *c;* those in B for S_1 and cytochrome *c;* and those in C for S_1, S_3, [^{3}H]GDP, and cytochrome *c*. The assay of S_1 and S_3 is described in Methods; the amounts are expressed for activity in 10-μl aliquots. [^{3}H]GDP was measured by counting 25-μl aliquots in Bray's scintillation fluid. Cytochrome *c* was detected by diluting aliquots in H_2O and measuring absorbancy at 409 mμ.

and extent of guanine nucleotide exchange by PEP and pyruvate kinase was excluded. According to this, these reagents would phosphorylate complex III to S_3–GTP complex and S_1 would promote then the exchange of free with complex-bound GTP. To test this alternative a reaction mixture including S_3–[^{3}H]GDP complex, PEP, and pyruvate kinase (in the amounts as in a reaction mixture in Figure 1B) as well as [^{3}H]GTP (in the amount as [^{32}P]GTP in a reaction mixture in Figure 1B) were incubated at 0° for 7 min. The fact that after this incubation less than 13% of the guanine nucleotide bound to S_3 was identified as [^{3}H]GTP, whereas after one in the presence of S_1 60%, rules this alternative out. This result indicates that apparently PEP and pyruvate kinase either do not phosphorylate GDP bound in complex III to GTP or at least do it slowly in comparison to the rate of the exchange.

S_1 Promotes the Release of GDP from Complex III. To elucidate the intermediate steps in the conversion of complex III to S_3–GDP complex we tested if S_1 also promotes a release of GDP from complex III in the absence of GTP. The curves in Figure 2 indicate that this is the case (see also Miller and Weissbach, 1970b; Waterson *et al.*, 1970). The same amount of S_1 results in the release of more GDP in the Sephadex assay than in the Millipore assay. This is probably due to the fact that (i) both assays reflect a competition for binding to S_3 between GDP and S_1; (ii) total GDP (part in complex III, part free) and S_1 are present during the filtration through Millipore in the ratio in which they are added to the incubation mixture; (iii) during gel filtration on Sephadex G-25 any GDP released from complex III is included in the gel and thereby separated from the excluded S_3, whereas S_1 and S_3 are both excluded and thus move together. This leads to a decrease in the GDP:S_1 ratio in the excluded fraction in which S_3 is eluted.

The fact that obtaining 80% release of GDP from complex III requires not much more than an equivalent amount of S_1 (8.5 pmoles of S_1 to 5.8 pmoles of complex III) is consistent with the possibility that S_1 may form a complex with S_3. (It should be noted that no binding of GDP by S_1 was detected in either the Millipore or Sephadex assay: G. B., unpublished data.)

Reaction of S_3–GDP Complex with S_1 Results in the Formation of an S_1–S_3 Complex and Release of GDP. It was noted in the Methods section that complex III is presumably identical with S_3–GDP complex and the separate designations complex III and S_3–GDP complex are used only to specify the way in which each is prepared. Complex III is isolated as an intermediate in the peptide chain elongation cycle whereas S_3–GDP complex is obtained essentially by incubating S_3 (which contains some bound GDP) with excess GDP and removing the free GDP thereafter.

After having performed the experiments in Figures 1 and 2 with complex III the experiment in Figure 2 was repeated with S_3–GDP complex. No difference was detected between the

TABLE I: Dissociation of S_1–S_3 Complex by GTP: Analysis of the Guanine Nucleotides Bound to S_3 in the Resulting S_3–Guanine Nucleotide Complex.[a]

Components in the Reaction Mixture Analyzed	Total Amount of Guanine Nucleotide Retained on Millipore Filter (cpm)	% of Guanine Nucleotides Retained on Millipore Filter	
		GTP	GDP
S_1–S_3	170	Not analyzed	
S_1–S_3 + [^{3}H]GTP, 93 pmoles	1616	79	21
S_1–S_3 + [^{3}H]GTP, 930 pmoles	5050	88	12
[^{3}H]GTP, 93 pmoles	69	Not analyzed	
[^{3}H]GTP, 930 pmoles	957	98	2

[a] S_1–S_3 complex was prepared by incubation of S_1 (12.6 μg), S_3 (11.7 μg), and [^{3}H]GDP (870 pmoles; specific activity 390 Ci/mole), and subsequent removal of [^{3}H]GDP by gel filtration (for details see the legend to Figure 3). The excluded fractions in the gel filtrate (containing S_1–S_3 complex and a very small amount of S_3–GDP complex) were pooled (total volume 0.33 ml) and divided into 0.05-ml aliquots. Two control aliquots (A and B) were not supplemented; two (C and D) were supplemented each with 93 pmoles and two (E and F) each with 930 pmoles of pretreated [^{3}H]GTP (specific activity approximately 1160 Ci/mole). After incubating the reaction mixture at 0° for 15 min each was filtered through a Millipore filter (HA13). The filters on which aliquots A, C, and E were filtered were counted (to determine the total amount of guanine nucleotides retained) and the filters on which aliquots B, D, and F were filtered were extracted with 0.1 ml of 0.05 M EDTA (pH 7.0) (Cooper and Gordon, 1969), and analyzed for GDP and GTP by chromatography on PEI (Randerath and Randerath, 1964). The data indicate that over 80% of the [^{3}H]GTP retained on the Millipore filter was in complex with S_3.

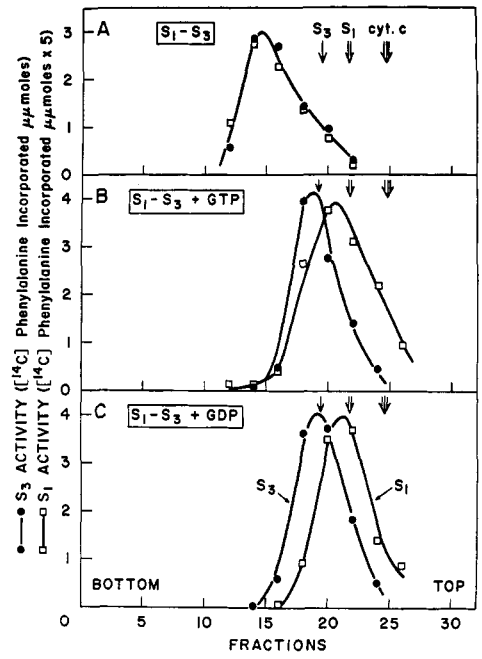

FIGURE 4: The S_1–S_3 complex is dissociated by GTP or GDP. S_1–S_3 complex was prepared by incubating S_3 (15.6 μg), S_1 (12.6 μg), and [^{3}H]GDP (800 pmoles) in 0.155-ml final volume at 30° for 10 min. The reaction mixture was fractionated by gel filtration on Sephadex G-25 (as described in the legend to Figure 3) to separate the S_1–S_3 complex in the excluded volume from the [^{3}H]GDP in the included volume. [^{3}H]GDP was included in the above reaction mixture to demonstrate that the amount of S_1 added was sufficient to bind most of the S_3 in an S_1–S_3 complex. The fact that the amount of [^{3}H]GDP excluded (*i.e.*, in the form of S_3–GDP complex) corresponded to only 27% of the amount of S_3 in the reaction mixture bore out this expectation. The excluded fractions containing S_1–S_3 complex were pooled (total volume 0.42 ml). One aliquot was supplemented with pretreated GTP (500 pmoles; B), a second with GDP (600 pmoles; C), and a third was not supplemented, except with buffer (A). After incubation at 0° for 10 min (to allow for the reaction of GTP and GDP with the S_1–S_3 complex) each reaction mixture was supplemented with a colored marker (cytochrome *c*, 0.3 mg) and layered over a sucrose gradient, as described in the legend to Figure 3, except that the gradient in Figure 4B contained 5 μM pretreated GTP and that in Figure 4C, 5 μM GDP. After centrifugation (see the legend to Figure 3) the gradients were collected in 31–32 fractions. S_1 and S_3 were assayed in the fractions by their polymerization activity (see Methods). The amounts are expressed for activity in 10-μl aliquots. The amount of cytochrome *c* is given in the legend to Figure 3. The position of S_3–GDP complex (single arrow), S_1 (double arrow), and cytochrome *c* (triple arrow) in the gradient is indicated in the figure. S_3–GDP complex and S_1 served as markers and were centrifuged in separate gradients.

reaction with complex III and that with S_3–GDP complex. Consequently since it is much easier to prepare S_3–GDP complex than complex III, all subsequent experiments were performed with S_3–GDP complex.

The curves in Figure 3 reflect the formation of S_1–S_3 complex in a reaction between S_3–GDP complex and S_1 and the release of GDP. The conclusion about the existence of the S_1–S_3 complex is based on the finding that S_1 and S_3 activities are sedimenting together (peak in fraction 14 and Figure 3C) and faster than either S_3–GDP complex tested in the absence of S_1 (peak in fraction 17 in Figure 3A) or S_1 tested in the absence of S_3–GDP complex (peak in fractions 18–19 in Figure 3B) (see also Miller and Weissbach, 1969; Lucas-Lenard *et al.*, 1969). Data on the promotion of the release of GDP from complex III (and from S_3–GDP complex) by S_1 was presented in the previous section. The curves in Figure 3 also demonstrate this phenomenon. Thus GDP cosediments with S_3 in the S_3–GDP complex (peak in fraction 17 in Figure 3A). However GDP does not cosediment with S_1–S_3 complex (Figure 3C). The small amount of GDP sedimenting with a peak in fraction 17 in this figure (about 10% of that sedimenting in the same fraction in Figure 3A) indicates that the conversion of S_3–GDP complex to S_1–S_3 complex was not complete; about 10% of the S_3–GDP complex persisted.

S_1–S_3 Complex Is Dissociated by GTP Resulting in the Formation of an S_3–GTP Complex. The experiments presented so far reveal that (i) S_1 promotes the formation of S_3–GTP complex from S_3–GDP complex and GTP; (ii) S_1 forms an S_1–S_3 complex in a reaction with S_3–GDP complex. These findings make it logical to test if addition of GTP to S_1–S_3 complex (i) will cause dissociation of the complex; and (ii) will result in S_3–GTP complex formation. The curves in Figure 4 and the data in Table I reveal that this is the case. If S_1–S_3 complex is sedimented through a sucrose gradient then S_1 and S_3

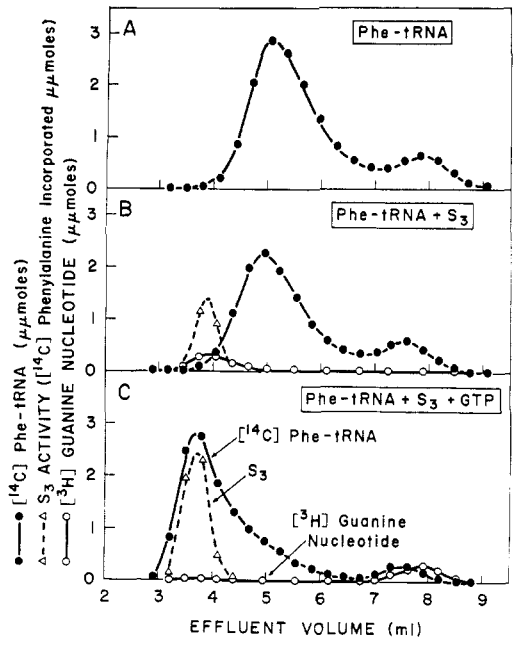

FIGURE 5: Binding of Phe-tRNA to S_3 requires GTP. The experiment was performed in two steps. First, a solution containing [^{14}C]-Phe-tRNA and S_3, free of guanine nucleotides, was prepared. Note that the incubation mixture for preparing this solution included [^{3}H]GTP (see Methods). The Mg^{2+} of this solution was adjusted to 14 mM. An aliquot (0.14 ml) of this solution was supplemented with unlabeled GTP (3000 pmoles; C); a second aliquot (0.14 ml; B) was not. Each aliquot was incubated at 30° for 2 min and analyzed by gel filtration on a Sephadex G-100 column (volume 8 ml, equilibrated with standard buffer). [^{14}C]Phe-tRNA was analyzed similarly (A). [^{3}H]Guanine nucleotides and [^{14}C]Phe-tRNA were measured by counting 0.2 ml aliquots of the fractions in 10 ml of Bray's scintillation fluid. S_3 was determined by its polymerization activity (see Methods). The small ^{14}C peak centering at about 7- to 8-ml effluent volume is phenylalanine which became released from the Phe-tRNA. It can be seen that the excess unlabeled GTP (C) displaces the small amount of [^{3}H]guanine nucleotide from the excluded fraction (B).

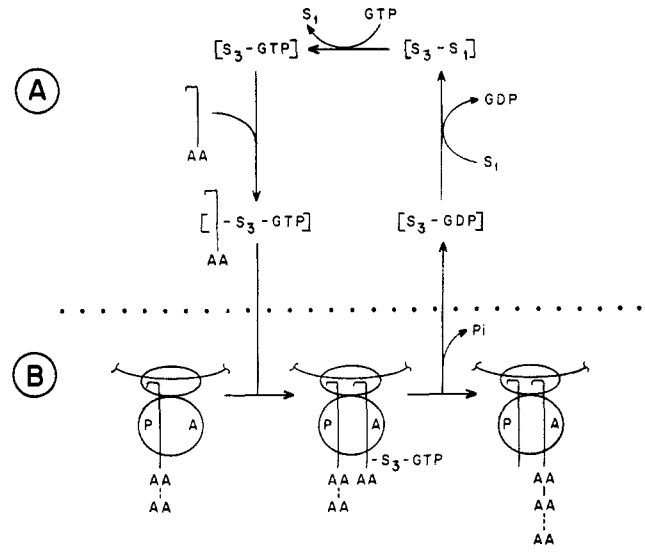

FIGURE 6: Function of S_1 in peptide chain elongation. The small oval shape represents the small ribosomal subunit, the large oval shape the large ribosomal subunit. P and A are hypothetical tRNA binding sites on the ribosome. The gallows shape stands for tRNA. The curved line bisecting the small ribosomal subunit represents mRNA. (A) Steps occurring away from the ribosome. (B) Steps occurring on the ribosome. (For further details, see the text.)

activities cosediment (Figure 4A). Incubation of S_1–S_3 complex with GTP and sedimenting it through a sucrose gradient including GTP result in the dissociation of S_1 and S_3; the two activities sediment separately and each of them slower than the S_1–S_3 complex (Figure 4B). The fact that GDP can be substituted for GTP in dissociating the S_1–S_3 complex (Figure 4C) indicates that the reaction of S_3–GDP complex with S_1 resulting in S_1–S_3 complex formation and the release of free GDP is reversible (Miller and Weissbach, 1969; Lucas-Lenard *et al.*, 1969).

When S_1–S_3 complex is dissociated with limiting amounts of GTP, then 79%, when dissociated with an excess of GTP, then 88%, of the guanine nucleotide bound in the resulting S_3–guanine nucleotide complex was found to be GTP, the rest being GDP (Table I). (Apparently either the S_1 preparation or the S_3 or both have a low level of GTPase activity.)

The reaction between S_1–S_3 complex and GTP resulting in S_3–GTP complex formation and release of S_1 is reversible. This conclusion is based on an experiment in which S_1 added to S_3–GTP complex caused release of GTP from the complex. (The experiment was performed according to the description of the Millipore assay in the legend to Figure 2 except that S_3–GTP complex was substituted for complex III; data not shown.)

Reaction of S_3–GTP Complex with AA-tRNA Resulting in AA-tRNA–S_3–GTP Complex Formation. The rate of binding of Phe-tRNA to S_3–GTP complex (both in the absence of S_1 and its presence) is so fast (essentially complete in less than 15 sec under our conditions) that we were unable to measure the kinetics (see also Weissbach *et al.*, 1970a). Thus we could not determine if S_1 is promoting this reaction or not.

Binding of Phe-tRNA to S_3 Requires GTP. It was established earlier that purified S_3 and AA-tRNA do not form a complex detectable in either the Millipore or the Sephadex assay (Skoultchi *et al.*, 1968; Shorey *et al.*, 1969). The finding that purified T_u (S_3) preparations contain bound GDP (Miller and Weissbach, 1970a) prompted us to reinvestigate the problem with S_3 free of guanine nucleotides. The curves in Figure 5A,B reveal that in the absence of GTP (i) Phe-tRNA is eluted on gel filtration in the same fraction (peak at 5.4-ml effluent volume) in the absence as in the presence of S_3 and (ii) S_3 is eluted (peak fraction at 3.8 ml effluent volume) in a different fraction. In the presence of GTP however, the peaks of S_3 and of Phe-tRNA are eluted in the same fraction (3.8-ml effluent volume in Figure 5C). These results indicate that GTP is required for forming a complex including S_3 and an AA-tRNA.

Is S_1 a Kinase Phosphorylating S_3–GDP Complex to S_3–GTP Complex? A priori it would be possible that (i) the conversion of S_3–GDP complex to S_3–GTP complex *via* release of GDP and binding of GTP as catalyzed by S_1 is an artefact and (ii) the physiological pathway is the phosphorylation of S_3–GDP complex to S_3–GTP complex by S_1 with GTP serving as a source of the phosphoryl residue transferred.

This possibility was tested in the following way. An S_3–[^{3}H]GDP complex was prepared (as described in the legend to Figure 3) and aliquots containing this were incubated with unlabeled pretreated GTP (0.36 mM) and (without and) with different S_1 preparations at 30° for 10 min. As a control, experiments were performed in which [^{3}H]GDP was substituted for S_3–[^{3}H]GDP complex. (It was ascertained that the pretreated GTP preparation was free of pyruvate kinase activity.) The reactions were stopped by adding formic acid (final con-

centration 1 M) and the [^{3}H]guanine nucleotides were analyzed for GTP and GDP by chromatography on PEI sheets (Randerath and Randerath, 1964).

Different preparations of purified S_1 (used in equal amounts of polymerizing activity) were found to exhibit a wide range of kinase activities as reflected in the conversion of 1.5–93% of the initial [^{3}H]GDP to [^{3}H]GTP. The simultaneous addition of 3′,5′-cAMP, 3′,5′-cGMP, ATP, CTP, or UTP in concentrations from 0.25 to 0.35 mM had no effect.

Less [^{3}H]GTP was formed from S_3–[^{3}H]GDP complex than [^{3}H]GDP exchanged: *e.g.*, in one experiment 79% of the ^{3}H counts was released from S_3–[^{3}H]GDP complex but only 41% of the [^{3}H]GDP was converted to [^{3}H]GTP. Kinase activity was also found by incubating [^{3}H]GDP with GTP and S_1 in the absence of S_3 and added S_3 had no effect on the rate of [^{3}H]GTP formation.

ATP could be substituted for GTP as a source of the phosphoryl residue transferred to [^{3}H]GDP and it is known that ATP does not substitute for GTP in AA-tRNA–S_3–GTP complex formation (Skoultchi *et al.*, 1968).

All these observations are consistent with the view that the kinase activity found is due to contaminants in the purified S_1 preparation. They make it unlikely that the physiological reaction catalyzed by S_1 could be the phosphorylation of the [^{3}H]GDP in the S_3–[^{3}H]GDP complex.

Discussion

Evidence for the involvement of S_1 in the formation of AA-tRNA–S_3–GTP complex from S_3–GDP complex, GTP, and AA-tRNA was provided in a previous communication (Waterson *et al.*, 1970). The results presented in this study are consistent with the following intermediate steps in this pathway: S_1 causes release of GDP from the S_3–GDP complex forming an S_1–S_3 complex. This reacts with GTP, S_1 is released, and an S_3–GTP complex is formed. The S_3–GTP complex binds AA-tRNA forming an AA-tRNA–S_3–GTP complex (Figure 6A). Apparently each of the steps in this pathway is reversible. The intermediate steps in the other half of the same cyclic pathway, *i.e.*, the formation of S_3–GDP complex from AA-tRNA–S_3–GTP complex, are shown in Figure 6B (Skoultchi *et al.*, 1970). The AA-tRNA–S_3–GTP complex is bound to a ribosome–mRNA complex (carrying peptidyl-tRNA in the P site), thereafter the GTP is cleaved, an S_3–GDP complex and P_i are released, and the peptidyl residue is released from its linkage to tRNA and forms a peptide linkage with the α amino group of the AA-tRNA attached at the A site of the ribosome. The utilization of the AA-tRNA–S_3–GTP complex pulls the reversible reactions in Figure 6A toward the production of this complex. The scheme in Figure 6A confirms an identical scheme proposed first by Weissbach and his associates (1970a) on the basis of studies with factors from *E. coli*. This scheme accounts for the fact that Tu (S_3) and Ts (S_1) can be isolated both in the form of a Ts–Tu (S_1–S_3) complex as well as in the form of separate factors (Nishizuka and Lipmann, 1966; Lucas-Lenard and Lipmann, 1966; Parmeggiani, 1968; Miller and Weissbach, 1970a; Kawakita *et al.*, 1971; Lockwood *et al.*, 1971).

The experiments on the dissociation of S_3–GDP complex by S_1 and the conversion of S_3–GDP complex to S_3–GTP complex and to AA-tRNA–S_3–GTP complex were performed both with complex III (isolated as an intermediate of this pathway) and with S_3–GDP complex (obtained by incubating purified S_3 with GDP). The fact that complex III and S_3–GDP complex behaved in an identical fashion may indicate that the two complexes are identical (see also Waterson *et al.*, 1970).

The most remarkable feature of the role of S_1 is that at least until the present there is no indication that S_1 is involved in either the cleavage or formation of any covalent linkage. Thus S_1 is apparently not a kinase phosphorylating S_3–GDP complex to S_3–GTP complex. Moreover the substrates and products of S_1 function, the S_3–guanine nucleotide complexes, have characteristics which are consistent with (though do not prove) the view that there is no covalent linkage between S_3 and either GDP or GTP in them. The complexes fall apart upon removal of Mg^{2+} from the reaction mixture (Weissbach *et al.*, 1970a) or on acidification with 1 M formic acid and GTP can be recovered from S_3–GTP complex (data not shown). The need for a separate protein, *i.e.*, S_1, in promoting the dissociation of S_3–GDP complex may be the consequence of the high affinity of S_3 for GDP.

Acknowledgments

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Interactions between Human Translocation Factor, Guanosine Triphosphate, and Ribosomes*

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ABSTRACT: Incubation of the human translocation factor TF II with [^{14}C]GTP and ribosomes results in the formation of a high molecular weight [^{14}C]GTP complex which is retained on nitrocellulose filters and can also be recovered in the exclusion peak after gel filtration through Sephadex G-25. The guanosine nucleotide present in this complex appears to be GDP. Despite extensive purification, the TF II protein fraction alone reveals a slight GTP binding activity. In addition to promoting the binding of [^{14}C]GTP to the ribosome, TF II itself is bound to the ribosome in the presence of a guanosine nucleotide. GDP can substitute for GTP in the reaction leading to the binding of TF II to ribosomes. The TF II–GDP–ribosome complex is stabilized by the presence of fusidic acid and its formation is inhibited by showdomycin. GTP increases the heat stability of TF II, but does not protect the factor against inactivation by showdomycin. The resistance of TF II to showdomycin is, however, increased when the factor is in the ribosome-bound state.

Studies in recent years have established many aspects of the mechanisms in polypeptide-chain elongation on the bacterial ribosome. Not all of these aspects have been explored to the same extent in mammalian polypeptide-chain elongation. The findings thus far suggest a basic analogy between the elongation mechanisms in bacterial and mammalian systems.

Regarding the translocation step, bacterial G factor as well as the corresponding mammalian factor TF II reveal some similar functional properties: both factors possess a ribosome-dependent GTPase activity (Nishizuka and Lipmann, 1966a; Felicetti and Lipmann, 1968) and transform bound AA-tRNA in the presence of GTP into a puromycin-reactive state (Haenni and Lucas-Lenard, 1968; Skogerson and Moldave, 1968a). Both systems are inhibited by fusidic acid, a specific translocation inhibitor (Tanaka *et al.*, 1968; Malkin and Lipmann, 1969). Furthermore, the bacterial G factor as well as the mammalian translocation factor possess SH groups, which are essential for their functional integrity (Nishizuka and Lipmann, 1966b; Kaziro *et al.*, 1969; Sutter and Moldave, 1966; Mosteller *et al.*, 1966).

Nonetheless, some differences seem to exist between the two systems. No interchangeability has thus far been observed between the bacterial and the mammalian translocation factors. Recently, however, it was demonstrated that the bacterial binding factor T can replace the mammalian factor in a cell-free system derived from rabbit reticulocytes (Krisko *et al.*, 1969). Furthermore, the mammalian translocation factor has been shown to be inactivated by diphtheria toxin in the presence of NAD (Collier, 1967), whereas bacterial pro-

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