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Peptide Chain Elongation: Indications for the Binding of an Amino Acid Polymerization Factor, Guanosine 5′-Triphosphate–Aminoacyl Transfer Ribonucleic Acid Complex to the Messenger–Ribosome Complex*

Arthur Skoultchi, Yasushi Ono, John Waterson, and Peter Lengyel

ABSTRACT: The intermediate steps in peptide chain elongation were studied in a cell-free system in which polyuridylic acid directs the formation of polyphenylalanyl transfer ribonucleic acid from phenylalanyl transfer ribonucleic acid. The system consists of guanosine 5′-triphosphate, salts, *Escherichia coli* ribosomes, and three amino acid polymerization factors (S_1, S_2, and S_3) from *Bacillus stearothermophilus*. (The factors from *B. stearothermophilus* correspond to the following factors from *E. coli:* S_1 to T_s, S_2 to G, and S_3 to T_u.) The incubation of S_1, S_3, guanosine 5′-triphosphate, and phenylalanyl transfer ribonucleic acid results in the formation of a S_3–guanosine 5′-triphosphate–phenylalanyl transfer ribonucleic acid complex (complex II). The incubation of complex II with a ribosome–polyuridylic acid–peptidyl transfer ribonucleic acid complex (actually acetylphenylalanyl transfer ribonucleic acid was used instead of peptidyl transfer ribonucleic acid) leads to the cleavage of guanosine 5′-triphosphate and the formation of an S_3–guanosine 5′-diphosphate complex and inorganic phosphate, as well as the synthesis of acetyldiphenylalanyl transfer ribonucleic acid. 5′-Guanylylmethylenediphosphonate, an analog of guanosine 5′-triphosphate which cannot be cleaved to guanosine 5′-diphosphate and inorganic phosphate, was substituted for guanosine 5′-triphosphate in an incubation mixture also containing S_1, S_3, phenylalanyl transfer ribonucleic acid, and the ribosome–polyuridylic acid–acetylphenylalanyl transfer ribonucleic acid complex. Under these conditions, approximately equimolar amounts of S_3, 5′-guanylylmethylenediphosphonate, and phenylalanyl transfer ribonucleic acid became bound and remained attached to ribosomes, and acetyldiphenylalanyl transfer ribonucleic acid was not formed. These results may reflect the transient existence of a ribosome-bound S_3–guanosine 5′-triphosphate–phenylalanyl transfer ribonucleic acid complex as an intermediate preceding acetyldiphenylalanyl transfer ribonucleic acid synthesis. The short life span of this hypothetical intermediate may be due to the rapid cleavage of the guanosine 5′-triphosphate molecule in it, which results in the release of an S_3–guanosine 5′-diphosphate complex and inorganic phosphate from the ribosome and acetyldiphenylalanyl–transfer ribonucleic acid formation.

The progress in the elucidation of the intermediate steps in peptide chain elongation is the consequence mainly of two developments: the isolation of the amino acid polymerization factors involved in the process, and the use of simple messenger RNAs, *i.e.*, homopolynucleotides in cell-free amino acid incorporating systems (Lipmann, 1969; Lengyel and Söll, 1969). *Escherichia coli* and *Pseudomonas fluorescens* were the first microorganisms in which three amino acid polymerization factors (T_u, T_s, and G) were described (Lucas-Lenard and Lipmann, 1966). The reported lability of one of these factors, T_u, prompted us to attempt the isolation of similar components from a different source. We purified from a thermophilic microorganism, *Bacillus stearothermophilus*, three such factors: S_1 corresponding to T_s, S_2 corresponding to G, and S_3 corresponding to T_u (Skoultchi *et al.*, 1968). Two of these are partially purified preparations (S_1, 30-fold; S_2, 70-fold) while the third, S_3 (purified 100-fold), moves as a single band on acrylamide gel electrophoresis. In a cell-free system consisting of S_1, S_2, and S_3 from *B. st.*,[1] thoroughly washed ribosomes from *E. coli*, GTP, and salts, polyuridylic acid directs the formation of polyPhe-tRNA from Phe-tRNA. The incubation of S_1 and S_3 with GTP and Phe-tRNA resulted in the formation of an S_3–GTP–Phe-tRNA complex (complex II) (Skoultchi *et al.*, 1968;

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[1] Abbreviations used are: *B. st.*, *Bacillus stearothermophilus;* GMPPCP, 5′-guanylylmethylenediphosphonate; polyPhe-tRNA, polyphenylalanyl transfer ribonucleic acid; Phe-tRNA, phenylalanyl-tRNA; ac-Phe-tRNA, acetylphenylalanyl-tRNA; ac-diPhe-tRNA, acetylphenylalanylphenylalanyl-tRNA; ac-polyPhe-tRNA, acetylpolyphenylalanyl-tRNA; AA-tRNA, aminoacyl-tRNA; RPA complex, ribosome–poly U–acetyl-Phe-tRNA complex; complex II, S_3–GTP–Phe-tRNA complex.

Ono *et al.*, 1968; Ravel *et al.*, 1968; Shorey *et al.*, 1969; Ertel *et al.*, 1968; Gordon, 1968). S_1 is presumably not part of complex II. On the basis of kinetic studies, it was established that complex II is an intermediate in the binding of aminoacyl-tRNA to the ribosome–messenger RNA–peptidyl-tRNA complex (Lucas-Lenard and Haenni, 1968; Ono *et al.*, 1969a). In the corresponding complex used in our studies, poly U served as messenger RNA and ac-Phe-tRNA as a convenient analog of peptidyl-tRNA (RPA complex) (Ono *et al.*, 1969b; Lucas-Lenard and Lipmann, 1967). Incubation of the RPA complex with complex II (or with the components required in forming complex II) results in (i) the binding of Phe-tRNA to the RPA complex, (ii) the reaction of part of the bound Phe-tRNA with the ac-Phe-tRNA to give ac-diPhe-tRNA (Ono *et al.*, 1969b; Lucas-Lenard and Haenni, 1968), and (iii) the cleavage of one GTP molecule (from complex II) for each Phe-tRNA molecule bound to the ribosome (Ono *et al.*, 1969a,b; Shorey *et al.*, 1969). The resulting ac-diPhe-tRNA does not react with puromycin indicating that it might be located in the aminoacyl-tRNA binding site (or A site) of the ribosome. The conversion of this ac-diPhe-tRNA into a puromycin reactive state, defined as translocation from the A site to the peptidyl-tRNA binding site (or P site), requires S_2 factor and further GTP. This GTP is cleaved to GDP and P_i (Ono *et al.*, 1969a; see also Traut and Monro, 1964; Haenni and Lenard, 1968; Erbe and Leder, 1968; Brot *et al.*, 1968; Pestka, 1968).

We wanted to test if the cleavage of GTP occurring after the attachment of Phe-tRNA to the RPA complex is a prerequisite for peptide bond formation. Therefore, in some experiments we substituted for GTP an analog of it, GMPPCP (Hershey and Monro, 1966), which has a methylene bridge instead of oxygen between the β and γ P atoms and consequently cannot be cleaved enzymatically to GDP and P_i. When S_1, S_3, and Phe-tRNA were incubated with the RPA complex in the presence of GMPPCP (instead of GTP) then Phe-tRNA became bound to the RPA complex but ac-diPhe-tRNA was not formed (Ono *et al.*, 1969b; Haenni and Lenard, 1968). This result was consistent with the need for GTP cleavage preceding peptide bond formation and suggested the existence of an intermediate formed after the binding of Phe-tRNA to the ribosome and persisting until peptide bond formation takes place. Such a hypothetical intermediate may consist of an RPA complex with Phe-tRNA, GTP, and perhaps S_3 attached.

In this communication we present data which may reflect on the existence of this intermediate. We find that ribosomes isolated after incubating the RPA complex with S_1, S_3, Phe-tRNA, and GMPPCP have bound to them approximately equimolar amounts of Phe-tRNA, S_3, and GMPPCP (but not ac-diPhe-tRNA), whereas ribosomes isolated after incubating the RPA complex with S_1, S_3, Phe-tRNA, and GTP have bound to them ac-diPhe-tRNA, (but no S_3, GTP, GDP, or P_i). Thus, while we have not been able to isolate an RPA–S_3–GTP–Phe-tRNA complex, we believe that its existence can be inferred from finding S_3 and GMPPCP attached to the ribosome along with Phe-tRNA. S_3 and GTP probably do become bound to the ribosome when Phe-tRNA is bound, but the resulting intermediate may have a short life span in consequence of the rapid cleavage of the GTP molecule in the complex into P_i and an S_3–GDP complex which are released from the ribosome. Since, however, GMPPCP cannot be cleaved into GDP and P_i, the corresponding intermediate formed in the presence of GMPPCP is stable.

It should be noted that results obtained from studies with a rat liver cell-free system also indicated that a factor involved in AA-tRNA binding to ribosomes can become attached to ribosomes in the presence of AA-tRNA. In contrast to our results, however, the association of the factor with ribosomes was observed in the presence of GTP, as well as with GMPPCP, although GTP was somewhat less effective than GMPPCP (Ibuki and Moldave, 1968).

Experimental Procedures

Components of the Cell-Free System. Phe-tRNA (unlabeled), [^{14}C]Phe-tRNA (specific activiy 409 μCi/μmole), [^{3}H]Phe-tRNA (specific activity 1650 μCi/μmole), and thoroughly washed ribosomes, all from *E. coli*, and amino acid polymerization factors (S_1 purified 30-fold, S_2 70-fold, and S_3 100-fold) from *B. st.* were prepared as described previously (Skoultchi *et al.*, 1968). Further details of the purification and characteristics of the *B. st.* factors will be published separately. (J. Waterson, G. Beaud, A. Skowhtchi, Y. Oho, and P. Lengyel manuscript in preparation) Phe-tRNA (unlabeled) was acetylated according to the procedure of Haenni and Chapeville (1966). GMPPCP was obtained from Miles Laboratories; [^{3}H]GDP (specific activity 1120 μCi/μmole) and [^{3}H]GTP (specific activity 800 μCi/μmole) from Schwarz BioResearch Inc.; [γ-^{32}P]GTP (specific activity 2370 μCi/μmole) from International Chemical Nuclear Co.; [^{14}C]GMP (specific activity 293 μCi/μmole) from Amersham-Searle Corp. [^{14}C]GMPPCP was prepared from [^{14}C]GMP according to the procedures of Moffatt and Khorana (1961), and Hershey and Monro (1966). Tetracycline was a gift from Dr. A. Laskin of the Squibb Institute for Medical Research. Sparsomycin was donated by the Cancer Chemotherapy National Service Center and Dr. G. S. Fonken of the Upjohn Company.

All reaction mixtures contained the following common components: 0.16 M NH_4Cl, 0.04 M Tris-HCl (pH 7.4), 0.01 M magnesium acetate, and 0.01 M dithiothreitol.

All Sephadex gel columns were equilibrated and eluted at 4° with a solution containing: 0.16 M NH_4Cl, 0.04 M Tris-HCl (pH 7.4), 0.01 M magnesium acetate, and 0.001 M dithiothreitol.

Preparation of the RPA Complex. Reaction mixture A (1 ml) contained, in addition to the common components, the following: ribosomes, 14.4 A_{260} units; poly U, 80 μg; and ac-Phe-tRNA (unlabeled), 3.75 A_{260} units. The reaction mixture was incubated at 30° for 60 min and then cooled to 0° (designated subsequently as incubated reaction mixture A).

Binding of Phe-tRNA to Ribosomes: Assay of [^{3}H]GTP, [γ-^{32}P]GTP and [^{14}C]GMPPCP Bound to Ribosomes. Reaction mixture B (1 ml) contained, in addition to the common components, some of the following if so indicated: S_1, 6.8 μg; S_3, 8 μg; and either GTP (^{3}H and γ-^{32}P labeled), 4 mμmoles, and [^{14}C]Phe-tRNA carrying 0.2 mμmole of phenylalanyl residues (12 A_{260} units) or [^{14}C]GMPPCP, 4 mμmoles, and [^{3}H]Phe-tRNA carrying 0.2 mμmole of phenylalanyl residues (20 A_{260} units). To 0.1 ml of incubated reaction mixture A containing the RPA complex, 0.1 ml of reaction mixture B was added. The resulting solution was incubated at 0° for 60 min and was fractionated by gel

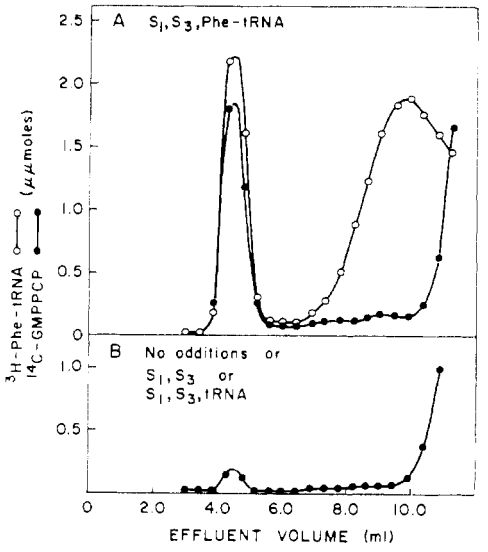

FIGURE 1: Binding of [^{14}C]GMPPCP and [^{3}H]Phe-tRNA to the RPA complex: gel filtration patterns. For details, see the section entitled Binding of Phe-tRNA to Ribosomes: Assay of [^{3}H]GTP, [γ-^{32}P]GTP, and [^{14}C]GMPPCP Bound to Ribosomes. [^{14}C]-GMPPCP was present in all the reaction mixtures. In the condition "No additions," reaction mixture B contained only [^{14}C]GMPPCP. In the condition "S_1, S_3" reaction mixture B contained only S_1, S_3 and [^{14}C]GMPPCP. In the condition "S_1, S_3, tRNA", 20 A_{260} units of discharged tRNA was substituted for [^{3}H]Phe-tRNA in reaction mixture B. The values shown represent the amounts in each fraction.

filtration on a 14-ml Sephadex G-150 column in order to separate the ribosomes with the substances bound to them from the unbound substances. Fractions of approximately 0.4 ml were collected. Aliquots (0.2 ml) of these fractions were counted in Bray's scintillator solution (Bray, 1960).

Binding of Phe-tRNA to Ribosomes: Assay of S_3 Bound to Ribosomes. Reaction mixture C (1 ml) contained, in addition to the common components, some of the following if so indicated: S_1, 6.8 μg; S_3, 8 μg; [^{14}C]Phe-tRNA carrying 0.2 mμmoles of phenylalanyl residues (12 A_{260} units), and either GMPPCP, 400 mμmoles, or GTP, 10 mμmoles. To 0.5 ml of incubated reaction mixture A containing the RPA complex, 0.5 ml of reaction mixture C was added. The resulting solution was incubated at 0° for 60 min and applied to a 14-ml Sephadex G-150 column. Fractions of approximately 0.5 ml were collected. Aliquots (0.1 ml) of these fractions were counted in Bray's scintillator solution in order to localize the excluded material, *i.e.*, RPA complex to which [^{14}C]Phe-tRNA had become bound. Four fractions of the excluded material, containing 85–90% of the RPA complex with [^{14}C]Phe-tRNA bound, were combined. These combined fractions were tested for the presence of S_3 in two ways: (1) *Polymerization Assay:* Reaction mixture D (1 ml) contained the common components (except that 0.03 M dithiothreitol was used) as well as the following components: [^{3}H]Phe-tRNA carrying 0.4 mμmole of phenylalanyl residue (40 A_{260} units); S_1, 0.85 μg; S_3, 0.5 μg; and GTP, 4 μmoles. To 0.4 ml of the combined fractions, 0.2 ml of reaction mixture D was added. The resulting solution was incubated at 30° to allow polyPhe-tRNA and ac-polyPhe-tRNA synthesis. At various times a 0.1-ml aliquot was removed, 2 ml of a 5% trichloroacetic acid solution was added, and the hot trichloroacetic acid insoluble material containing poly-Phe-tRNA and ac-polyPhe-tRNA was filtered on a Millipore filter and counted in a toulene-based scintillator solution. (2) *GDP Binding Assay:* To 1 ml of the combined fractions, 5 μl of a solution containing 5 mμmoles of [^{3}H]GDP was added. The resulting solution was incubated at 30° for 10 min in order to obtain the release of S_3 from ribosomes and then applied to a 14-ml Sephadex G-100 column. The [^{3}H]-GDP–S_3 complex was localized in the effluent by collecting 0.4-ml fractions and counting 0.2-ml aliquots of each in Bray's scintillator solution.

Preparation and Isolation of Complex II. The reaction mixture (1 ml) contained, in addition to the common components, the following components: Phe-tRNA (unlabeled), 32 A_{260} units; S_1, 5.4 μg; S_3, 5 μg; and GTP (^{3}H and γ-^{32}P labeled), 1 mμmole. This reaction mixture was incubated at 30° for 4 min cooled to 0°, and applied to a Sephadex G-25 column. Gel filtration was performed in order to separate complex II from free GTP. (The excluded fraction containing the S_3–GTP–Phe-tRNA complex and S_1 will be designated subsequently as complex II solution.)

Results

Binding of GMPPCP to Ribosomes: Dependence upon Poly U, Phe-tRNA, S_1, and S_3. Reaction mixtures containing components of the *in vitro* protein synthesizing system were analyzed by gel filtration. Gel filtration on Sephadex G-150 was used to separate the ribosomes with the substances bound to them from substances not bound to the ribosomes. A gel filtration pattern demonstrating this is shown in Figure 1. An incubated reaction mixture including RPA complex, S_1, S_3, Phe-tRNA, and GMPPCP was analyzed. The effluent volume of ribosomes on the column used was between 4 and 5.5 ml; smaller substances had larger effluent volumes. Some of the GMPPCP and Phe-tRNA were eluted between 4 and 5.5 ml of effluent volume indicating that a part of these compounds was bound to ribosomes (Figure 1A). The amount of GMPPCP bound to ribosomes decreased to 0.1 when Phe-tRNA was omitted from the incubation mixture, or when uncharged tRNA was substituted for it (Figure 1B).

Results of experiments, in which the effect of various substances on the binding of GMPPCP, GTP, and Phe-tRNA to ribosomes was studied by gel filtration, are summarized in Table I.

The data indicate that if the attachment of Phe-tRNA to the RPA complex is promoted by S_1, S_3, and GTP then the net amount of GTP bound is only a small fraction (about 10%) of the net amount of Phe-tRNA bound. This may be a consequence of the rapid cleavage of GTP occurring after the binding (Ono *et al.*, 1969a,b). Even if peptide bond formation between the bound Phe-tRNA and the ac-Phe-tRNA component of the RPA complex is blocked by the antibiotic sparsomycin (Lucas-Lenard and Haenni, 1968; Ono *et al.*, 1969b), the ratio of net GTP bound to net Phe-tRNA bound remains low. This result is consistent with the fact that sparsomycin does not inhibit the GTP cleavage (Ono *et al.*, 1969b). The small amounts of label bound to ribosomes from [γ-^{32}P]GTP and from [^{3}H]GTP show that neither of the two cleavage products of GTP (*i.e.*, GDP and P_i) remains attached to ribosomes.

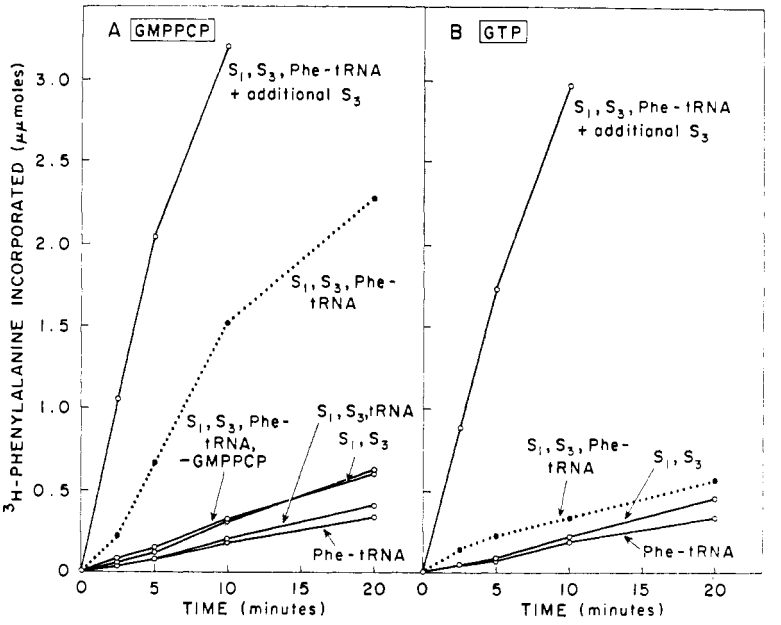

FIGURE 2: Binding of S_3 to the RPA complex: polymerization assay. For details see the section entitled Binding of Phe-tRNA to Ribosomes: Assay of S_3 Bound to Ribosomes; (1) Polymerization Assay. In the condition "S_1, S_3, Phe-tRNA and additional S_3," 1 ml of reaction mixture D was supplemented with 4 μg of S_3. In the condition "S_1, S_3, tRNA," 20 A_{260} units of discharged tRNA/ml was substituted for [^{14}C]Phe-tRNA in the reaction mixture C.

TABLE I: Binding of Phe-tRNA, GTP, and GMPPCP to the RPA Complex: Comparison of the Amounts Bound.[a]

Additions	[^{3}H]Phe-or [^{14}C]Phe-tRNA Bound to Ribosomes ($\mu\mu$moles)	[^{3}H]GTP Bound to Ribosomes ($\mu\mu$moles)	[^{14}C]GMPPCP Bound to Ribosomes ($\mu\mu$moles)	[γ-^{32}P]GTP Bound to Ribosomes ($\mu\mu$moles)
S_1, S_3, GMPPCP, Phe-tRNA	4.4 (3.2)		3.6 (3.3)	
S_1, S_3, Phe-tRNA	1.2			
S_1, S_3, GMPPCP			0.3	
S_1, S_3, GMPPCP, tRNA			0.4 (0.1)	
S_1, S_3, GMPPCP, Phe-tRNA, —poly U	0.1		0.3 (0)	
S_1, S_3, GMPPCP, Phe-tRNA, tetracycline	0.5		0.5 (0.2)	
GMPPCP, Phe-tRNA	0.9		0.3 (0)	
S_1, S_3, GTP, Phe-tRNA	3.9 (2.7)	0.4 (0.2)		0.1 (0)
S_1, S_3, GTP, Phe-tRNA, sparsomycin	4.0 (2.8)	0.6 (0.4)		0.1 (0)
S_1, S_3, GTP		0.2		0.1

[a] Conditions were the same as described in the legend to Figure 1. The values shown represent the sum of the amounts of the specified compounds which were present in those fractions eluted between 4.0 and 5.5 ml of the G-150 effluent (see Figure 1). The net amounts of Phe-tRNA bound to ribosomes (shown in parentheses) were obtained by subtracting the amount bound to ribosomes in the presence of S_1 and S_3, but in the absence of GMPPCP and GTP. The net amounts of GMPPCP or GTP bound to ribosomes (shown in parentheses) were obtained by subtracting the amount bound to ribosomes in the absence of Phe-tRNA. Where indicated: 20 A_{260} units of discharged tRNA (per milliliter) was substituted for [^{3}H]Phe-tRNA in reaction mixture B; poly U was omitted from reaction mixture A; 0.4 μmole of tetracycline-HCl/ml or 0.2 μmole of sparsomycin/ml was added to reaction mixture B.

If the attachment of Phe-tRNA to the RPA complex is promoted by S_1, S_3, and GMPPCP, then the net amounts of GMPPCP and Phe-tRNA bound are about equimolar. The GTP analog promotes the binding of Phe-tRNA in the presence of S_1 and S_3 to approximately the same extent as GTP does. Furthermore, the binding of GMPPCP to ribosomes occurs only in conditions in which Phe-tRNA is also bound. GMPPCP is not bound if the reaction mixture does not contain either poly U, or S_1 and S_3, or Phe-tRNA (uncharged tRNA is no substitute for Phe-tRNA). The antibiotic tetracycline which inhibits Phe-tRNA binding (see also Sarkar and Thach, 1968) also inhibits GMPPCP binding.

Binding of the S_3 Factor to Ribosomes: Dependence upon Poly U, Phe-tRNA, and GMPPCP. Difference between the Effect of GMPPCP and GTP on the Binding. Reaction mixtures containing RPA complex, Phe-tRNA, and various combinations of the substances required for binding Phe-tRNA to ribosomes were incubated and subsequently fractionated by gel

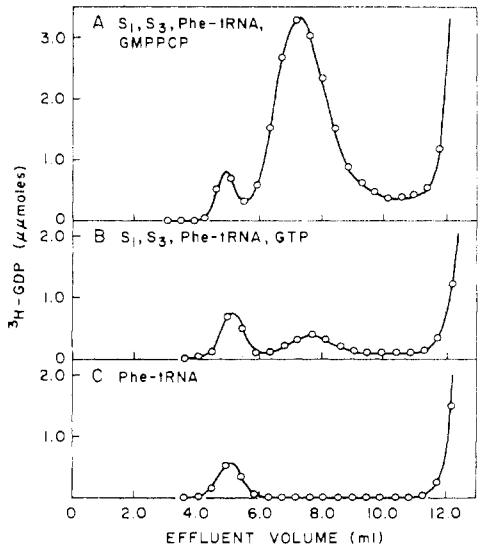

FIGURE 3: Binding of S_3 to the RPA complex: gel filtration patterns indicating the release of S_3 from ribosomes by incubation with [^{3}H]GDP. For details, see the section entitled Binding of Phe-tRNA to Ribosomes: Assay of S_3 Bound to Ribosomes; (2) GDP Binding Assay. The values shown represent the amount of [^{3}H]-GDP in each fraction. The values experimentally measured were multiplied by a factor (between 100/85 and 100/90) in order to correct for the fact that only 85–90% of the RPA complex with [^{14}C]Phe-tRNA bound was used in testing the amount of S_3 released.

TABLE II: Binding of S_3 to the RPA Complex: Comparison of the Amounts of S_3 and Phe-tRNA Bound.[a]

Additions	[^{14}C]Phe-tRNA Bound to Ribosomes ($\mu\mu$moles)	S_3 Released from Ribosomes by GDP ([^{3}H]-GDP–S_3 Complexes) ($\mu\mu$moles)
S_1, S_3, GMPPCP, Phe-tRNA	29.4 (19.9)	17.4
S_1, S_3, GTP, Phe-tRNA	30.0 (20.5)	1.8
S_1, S_3, GTP, Phe-tRNA, sparsomycin	28.8 (19.3)	2.1
S_1, S_3, Phe-tRNA	9.5	<0.2
S_1, S_3, GMPPCP		<0.2
S_1, S_3, GMPPCP, tRNA		<0.2
S_1, S_3, GMPPCP, Phe-tRNA, —poly U	0.3	<0.2

[a] Conditions were the same as in the legend to Figure 3. The values for [^{14}C]Phe-tRNA bound to ribosomes represent the amounts of [^{14}C]Phe-tRNA bound to ribosomes attached before the incubation with [^{3}H]GDP. The values for [^{3}H]-GDP–S_3 complexes represent the sum of the amounts of [^{3}H]GDP present in the fractions eluted between 6.0 and 9.0 ml of the effluent (see Figure 3). These values have been multiplied by a factor as described in the legend to Figure 3. The net amounts of [^{14}C]Phe-tRNA bound to ribosomes (shown in parentheses) were obtained by subtracting the amount of [^{14}C]Phe-tRNA bound to ribosomes in the absence of GMPPCP (additions: S_1, S_3, and Phe-tRNA in the table) (9.5 $\mu\mu$moles). Where indicated: 0.2 μmole of sparsomycin/ml was added to reaction mixture C; 20 A_{260} units of discharged tRNA/ml was added to reaction mixture C; poly U was omitted from reaction mixture A.

filtration on Sephadex G-150. The presence of S_3 factor on the isolated ribosomes was tested in two ways. One method consisted of supplementing the effluent fraction containing the RPA complex with all components required for the synthesis of polyPhe-tRNA (*i.e.*, [^{3}H]Phe-tRNA, GTP, S_1, and S_2) except S_3, and determining the capacity of the supplemented RPA complex to synthesize [^{3}H]polyPhe-tRNA. The dashed curve in Figure 2A demonstrates that an RPA complex which has been incubated with S_1, S_3, Phe-tRNA, and GMPPCP before gel filtration is capable of synthesizing [^{3}H]polyPhe-tRNA under these conditions. This indicates that the ribosomes have S_3 attached. On the other hand, if GTP is substituted for GMPPCP (in the reaction mixture in which the RPA complex is incubated before gel filtration), then the isolated and supplemented RPA complex is not able to synthesize polyPhe-tRNA (at least not to a greater extent than an RPA complex which had never been incubated with S_3; compare S_1, S_3, Phe-tRNA, and Phe-tRNA in Figure 2B). As a control, it was shown that such a complex can synthesize polyPhe-tRNA, however, if also supplemented with additional S_3 (Figure 2B). It should be noted that only that isolated RPA complex carries bound S_3 which has been incubated with S_1, S_3, Phe-tRNA, and GMPPCP. If Phe-tRNA or GMPPCP or S_1 and S_3 are omitted from the incubation mixture, or if uncharged tRNA is substituted for Phe-tRNA, S_3 cannot be found attached to ribosomes (Figure 2A).

The second method used to test for the presence of S_3 on the isolated ribosomes was based on the fact that S_3 can bind GDP (Skoultchi *et al.*, 1968; Gordon, 1967). The assay included an incubation of the ribosome-containing fraction of the gel filtrate with [^{3}H]GDP. This treatment resulted in the release of any S_3 bound to ribosomes. When the reaction mixture thus obtained was analyzed by gel filtration on Sephadex G-100, then the S_3 (which had been bound to ribosomes prior to the treatment with GDP) was recovered in the form of a [^{3}H]GDP–S_3 complex (Figure 3A). (We verified that the presumed [^{3}H]GDP–S_3 complex has S_3 activity by showing its effect in polyPhe-tRNA synthesis–data not shown.) If ribosomes isolated from a reaction mixture in which RPA complex was incubated with S_1, S_3, Phe-tRNA, and GMPPCP are treated with [^{3}H]GDP, they release a large amount of [^{3}H]GDP–S_3 complex (the fractions eluting between 6 and 9 ml in Figure 3A—see also Table II). The amount of [^{3}H]GDP–S_3 complex (calculated in $\mu\mu$moles of GDP) released from the ribosomes is approximately equimolar to the net amount of [^{14}C]Phe-tRNA bound to the ribosomes before the treatment with GDP (Table II). Ribosomes isolated from reaction mixtures in which RPA complex was incubated with S_1 and S_3, Phe-tRNA, and GTP release only 0.1 as much [^{3}H]GDP–S_3 complex as ribosomes isolated from similar incubation mixtures but containing GMPPCP instead of GTP (Figure 3 and Table II). These data seem to

indicate that S_3 (or at least the majority of it) has been released from such ribosomes before they were isolated. The amount of Phe-tRNA bound to ribosomes is not diminished, however, by substituting GTP for GMPPCP (Table II).

S_3 is not bound to ribosomes in the presence of GMPPCP under conditions in which there is no Phe-tRNA binding to ribosomes (*i.e.*, in the absence of poly U or in the absence of Phe-tRNA, even if uncharged tRNA is present). Nor is S_3 bound to ribosomes under conditions in which there is only nonenzymatic binding of Phe-tRNA (*i.e.*, in the presence of Phe-tRNA but with no GMPPCP) (Table II). Ribosomes, even if never incubated with S_1 and S_3, do bind a small amount of [^{3}H]GDP (eluted between 4 and 6 ml in Figure 3C). This accounts for the small peaks of [^{3}H]GDP eluted with the ribosome fraction in Figures 3A and B.

Reaction of Complex II with the RPA Complex: Formation of an S_3–GDP Complex Not Attached to Ribosomes. As reported in previous communications (Ono *et al.*, 1969a,b), the reaction of complex II with the RPA complex results in ac-diPhe-tRNA synthesis and the cleavage of the GTP molecule in complex II to GDP and P_i. The data presented in the preceding sections of this paper seem to indicate that after the reaction, the GDP and P_i formed, as well as the S_3 factor, can be found in the reaction mixture unattached to ribosomes. It was known that S_3 factor can form a complex with GDP though not with GMP (Skoultchi *et al.*, 1968). We wanted to establish if the S_3 component which is released from the complex II–RPA complex after GTP cleavage does form a complex with the GDP produced in the reaction. For this purpose complex II was prepared containing equimolar amounts of [^{3}H]GTP and [γ-^{32}P]GTP. This complex was incubated with RPA complex and the reaction mixture was thereafter analyzed by gel filtration on Sephadex G-150 in order to differentiate the GTP and GDP bound to S_3 (effluent volume approximately 5–10 ml in Figure 4B) from unbound GTP, GDP, and P_i (effluent volume approximately 10–15 ml in Figure 4B). The curves in Figure 4B show that: (i) very little of the GTP or of its cleavage products is bound to ribosomes; (ii) most of the [^{3}H]guanine moiety from the [^{3}H]GTP is bound to S_3; and (iii) most of the [γ-^{32}P]-phosphate moiety from the [γ-^{32}P]GTP is not bound to S_3. The fact that much more of the guanine moiety than of the γ-phosphate moiety is bound to S_3 indicates that the majority of the bound nucleotide is GDP. The small amount of [γ-^{32}P]-phosphate bound to S_3 suggests that a small portion of complex II did not react with the RPA complex. As a control, it was verified that if complex II is incubated in a reaction mixture containing all components of the RPA complex except ribosomes, then all of the [^{3}H]guanine moieties and all of the [γ-^{32}P]-phosphate moieties remain bound in the complex (Figure 4A). This is because under these conditions GTP is not cleaved. These data indicate the existence of an S_3–GDP complex formed after the reaction of complex II with the RPA complex, and confirms similar observations of Ravel and her associates with the *E. coli* system (Shorey *et al.*, 1969). It should be noted, however, that whereas the GDP moiety of the S_3–GDP complex of Shorey *et al.* (1969) was not exchangeable with GTP or GDP at 0°, the GDP of our complex was (data not shown). It remains to be established if the cause of this apparent discrepancy is that the reaction mixture used by Shorey *et al.* (1969) did not contain S_1 factor while ours did.

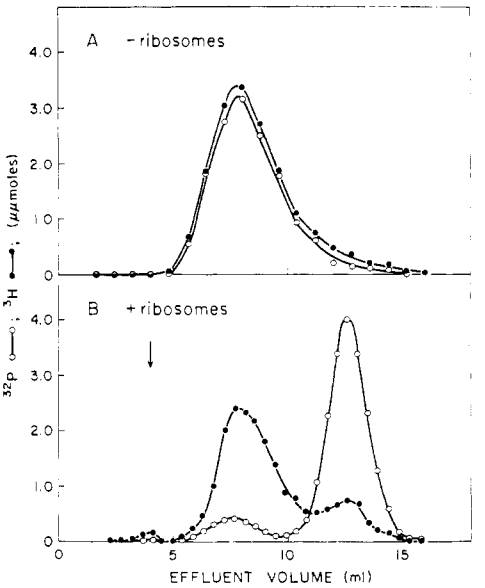

FIGURE 4: Formation of an S_3–GDP complex during the reaction of complex II with the RPA complex. To 0.25 ml of incubated reaction mixture A containing the RPA complex, 0.5 ml of complex II solution was added containing 30 $\mu\mu$moles of GTP labeled with ^{3}H and ^{32}P (in the γ position). The resulting solution was incubated at 0° for 20 min and subsequently analyzed on a 15-ml Sephadex G-150 column in order to differentiate GTP and GDP bound to S_3 from unbound GTP and GDP. Fractions of 0.5 ml were collected and 0.3-ml aliquots of these were counted in Bray's scintillator solution. In part A ribosomes were omitted from reaction mixture A. The vertical arrow in part B indicates the peak of A_{260} in the effluent. This is due to the elution of ribosomes.

Discussion

Complex II is the source of the aminoacyl-tRNA bound to the RPA complex (Ono *et al.*, 1969a; Lucas-Lenard and Haenni, 1968). The binding of S_3, Phe-tRNA, and GMPPCP to the RPA complex may indicate that in the course of protein synthesis, complex II (including its S_3 moiety) becomes bound to the RPA complex and remains attached until the cleavage of the GTP moiety. The following results are consistent with this view: complex II contains equimolar amounts of AA-tRNA and GTP (Ono *et al.*, 1968, 1969a), and equimolar amounts of GMPPCP and AA-tRNA are bound to the RPA complex. Furthermore, our data seem to indicate that the amount of S_3 bound to the RPA complex is also equimolar with that of GMPPCP and AA-tRNA bound. This conclusion is based on the assumption that the moles of [^{3}H]GDP bound to S_3, which is released by treatment of the RPA complex with [^{3}H]GDP, can be taken as equal with the moles of released S_3. Results of recent experiments seem to support the validity of this assumption (unpublished observations). A firmer support for the above conclusion, however, would be a direct determination of the amount of S_3 protein bound to the RPA complex in the presence of GMPPCP and AA-tRNA.

We believe that the results obtained in experiments with GMPPCP may serve as a basis for assuming the existence of the proposed complex II–RPA complex intermediate. It should be noted, however, that S_3 does recognize the difference between the GTP analog and GTP even prior to the

cleavage of GTP: the S_3–GTP–AA-tRNA complex (complex II) is sufficiently stable to persist during gel filtration. However, no S_3–GMPPCP–AA-tRNA complex could be detected when tested by gel filtration under the same conditions (unpublished observations). It would be desirable to provide proof for the existence of the proposed complex II–RPA complex intermediate by methods not involving the use of GTP analogs. A good proof, for example, would consist of inhibiting the cleavage of the GTP in the proposed complex II–RPA complex and demonstrating under these conditions that the complex is stable.

The isolation of an S_3–GDP complex as a product of the reaction of complex II with the RPA complex raises the question of what steps are needed for reutilizing this complex (or at least the protein moiety of it) by converting it into complex II.

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