THE BINDING OF AMINOACYL-tRNA AND POLY U TO A SOLUBLE FACTOR(S) EXTRACTED FROM RIBOSOMES

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SUMMARY: Soluble proteins have been obtained from Escherichia coli ribosomes which have the ability to bind N-acetyl-Phe-tRNA and poly U. In addition, an interaction of a Phe-tRNA-Tu-GTP complex with the soluble extract has been demonstrated.

Although considerable information has been obtained concerning the isolation and mechanism of action of the soluble factors required for polypeptide synthesis, little is known about the factors on the ribosome which are needed for protein synthesis.[1] Attempts have been made to separate and identify the proteins associated with bacterial ribosomes. For example, the initiation factors are readily extracted from the ribosomes with high concentrations of NH_4Cl (1). The elegant studies of Nomura and coworkers (2,3) have resulted in the separation of the proteins from the 30s subunit and subsequent reconstitution of a fully active 30s particle. In addition, this group has been able to identify the protein on the 30s subunit responsible for streptomycin resistance. Spitnik-Elson and coworkers (4,5) have used a variety of techniques to remove and solubilize essentially all of the proteins on the ribosome. However, in general, there has been little success with regard to obtaining soluble proteins possessing activity in the partial reactions associated with protein synthesis. For example, most mechanisms describing the events involved in peptide bond formation refer to as yet unidentified "donor" and "acceptor" sites on the ribosome. In addition, peptidyl synthetase, the enzyme that catalyzes the synthesis of the peptide bond, is an integral part of the ribosome (6), and GTP hydrolysis, associated with both the action of factor G (7) and the Tu dependent

[1] For a recent review of protein synthesis see Cold Spr. Harb. Symp. Quant. Biol. 23 (1969).

binding of aminoacyl-tRNA (AA-tRNA) to the ribosome (8-10), depends on ribosomal factors. In all the above examples the inability to solubilize the required ribosomal factors has hampered studies on their nature and function.

It has now been possible to extract ribosomes with a solution containing 2 mM Mg^{++} and 1 M KCl and obtain a soluble extract which interacts with various RNA species. In this report preliminary studies are presented on the reaction of Phe-tRNA, N-acetyl-Phe-tRNA, and an AA-tRNA-Tu-GTP complex with this soluble preparation. In addition, this soluble preparation also binds poly U.

MATERIALS AND METHODS: ^{14}C-Phenylalanine (sp. act. 360 μc/μmole) and ^{3}H-GTP (sp. act. 1.2 C/mmole) were obtained from New England Nuclear Corp.; γ-^{32}P-GTP from International Chemical and Nuclear Corporation, and ^{3}H-polyuridylic acid from Miles Research Laboratories. E. coli unfractionated deacylated tRNA was obtained from General Biochemicals, and ^{14}C-Phe-tRNA was prepared using unfractionated tRNA by the procedure of Conway (11). Purified $tRNA^{Phe}$ (50% pure) was prepared by Dr. John Hachmann essentially as described by Gillam et al. (12). Unfractionated ^{14}C-Phe-tRNA was converted to N-acetyl-^{14}C-Phe-tRNA by the procedure of Haenni and Chapeville (13). A homogeneous Tu preparation was prepared by Dr. David Miller as described elsewhere (14,15). E. coli MRE 600 cells were purchased from Grain Processing Corporation, and a cell-free extract was prepared by disrupting the cells in an equal volume (w/v) of a buffer containing Tris-Cl, pH 7.8, 50 mM; $MgCl_2$, 14 mM; β-mercaptoethanol, 6 mM, and 4 μg/ml of DNase. This preparation was centrifuged at 30,000 x g for 30 min, and the supernatant solution was then centrifuged for 2 hrs at 150,000 x g to isolate the ribosomes. The ribosomes were washed four times with a solution containing Tris-Cl buffer, pH 7.4, 10 mM; $MgCl_2$, 10 mM; NH_4Cl, 0.5 M; and DTT, 1 mM, except for the last wash, in which case the NH_4Cl concentration of the buffer was raised to 1 M. The purified ribosomes were stored in a buffer solution containing Tris-Cl, pH 7.4, 1 mM; Mg acetate, 10 mM; and DTT, 1 mM. To obtain a soluble protein extract these ribosomes were then suspended essentially as described by Spitnik-Elson and Atsmon (4) in a

buffer containing Tris-Cl, pH 7.4, 10 mM; $MgCl_2$, 2 mM; and KCl, 1 M, to obtain a final ribosome concentration of 1 mg/ml (1 mg = 14.4 A_{260}). This suspension was left at 4^o for 4-16 hrs and then centrifuged at 175,000 x g for 2 hrs, and the supernatant fraction used as the source of the soluble extract (ribosome extract) after concentrating the solution by ultrafiltration in a Diaflo apparatus (Amicon Corp.). This extract had a protein/RNA ratio of 3.95 as compared to 0.67 for the ribosomes before extraction. The formation of the AA-tRNA-Tu-GTP complex from Tu-GTP and AA-tRNA was assayed by a nitrocellulose filter assay. The incubations were performed at 37^o for 10 min and contained in a total volume of 0.5 ml, 600 units of Tu, 300-400 pmoles of purified ^{14}C-Phe-tRNA, 50 μg of pyruvate kinase, 6 x 10^{-3} M phosphoenolpyruvate, 0.01 M $MgCl_2$, 0.05 M Tris-Cl buffer, pH 6.5, and 5 mμmoles γ-^{32}P-^{3}H-GTP. The incubation mixtures were then passed through a stack of two nitrocellulose filters (0.45 μ Millipore Corporation) which separated the Tu-GTP (retained by the filter) from AA-tRNA-Tu-GTP (in the filtrate). The filters were washed with 0.5 ml of a buffer solution containing 10 mM $MgCl_2$, NH_4Cl, and Tris-Cl, pH 7.4. The wash and the filtrate were combined and used for the experiments employing the AA-tRNA-Tu-GTP complex. It should be noted that the solution containing the ternary complex was essentially free of any Tu-GDP and Tu-GTP, but possibly contained small amounts of unreacted Phe-tRNA (the Phe-tRNA was used in limiting amounts and was essentially all present as part of the ternary complex), and all of the unreacted γ-^{32}P-^{3}H-GTP. The assay for factors present in the ribosomal extract which interacted with AA-tRNA, poly U, and the AA-tRNA-Tu-GTP complex was based on retention of these substances on a nitrocellulose filter in the presence of the ribosomal extract.

The incubation for the binding of N-acetyl-Phe-tRNA or Phe-tRNA was carried out for 5 min at 0^o and contained in a total volume of 0.05 ml: ribosomal extract; Tris-Cl buffer, pH 7.4, 10 mM; $MgCl_2$, 15 mM; NH_4Cl, 100 mM; KCl, 200 mM; β-mercaptoethanol, 12 mM, and about 10 pmoles of unfractionated N-acetyl-Phe-tRNA or Phe-tRNA (0.44 A_{260}). The reaction mixture was then

diluted with 2 ml of a buffer containing Tris-Cl, pH 7.4, 50 mM; $MgCl_2$, 12 mM; and NH_4Cl, 160 mM, filtered through a nitrocellulose filter, and washed with 4 ml of the same buffer. The incubation for the binding of the AA-tRNA-Tu-GTP complex was carried out for 2 min at 0^o in a final volume of 0.05 ml. This incubation mixture contained: ribosomal extract, Tris-Cl buffer, pH 7.0, 20 mM; NH_4Cl, 30 mM; KCl, 25 mM; $MgCl_2$, 10 mM, and about 19 pmoles of the AA-tRNA-Tu-GTP complex. At the completion of the incubation the reaction mixture was diluted and passed through a nitrocellulose filter, and the filter was washed as described above. Poly U binding was assayed by incubating the ribosomal extract for 5 min at 0^o in 1 ml of a buffer containing Tris-Cl, pH 7.8, 10 mM; KCl, 50 mM; $MgCl_2$, 14 mM; and ^{3}H-poly U, 2.5 μg. The incubation mixture was then passed through a nitrocellulose filter [these filters had been previously treated with KOH as described by Smolarsky and Tal (16)] and washed with 8 ml of the incubation buffer. The filters from all the experiments were assayed for radioactivity in a Beckman LS-100 liquid scintillation counter after being dissolved in 10 ml of a counting solution described by Bray (17).

TABLE I

Binding of N-Acetyl-^{14}C-Phe-tRNA and ^{14}C-Phe-tRNA to a Ribosomal Soluble Factor

System	Poly U	Acylated-tRNA bound
		pmoles
N-acetyl-Phe-tRNA	-	1.76
	+	1.16
Phe-tRNA	-	0.76
	+	0.49

The assay conditions are described in the text. Each incubation contained 26 μg of the ribosomal extract, and where indicated, 5 μg of poly U.

RESULTS: A 1.0 M KCl soluble extract of ribosomes, prepared as described in Methods, interacted with N-acetyl-Phe-tRNA at 0^{o} to yield a complex which was retained on a nitrocellulose filter. As seen in Table I, binding was also observed with Phe-tRNA, and the binding of both tRNA's was inhibited by the addition of poly U to the incubation reactions. The latter result is in contrast to what is observed with intact ribosomes where the binding of AA-tRNA to ribosomes is dependent on the presence of messenger RNA. The reaction with N-acetyl-Phe-tRNA was complete in 3 min and was dependent on the concentration of the extract (Fig. 1). No binding of the amino acid occurred if the AA-tRNA was deacylated with alkali prior to binding. The factor in the extract was 65%

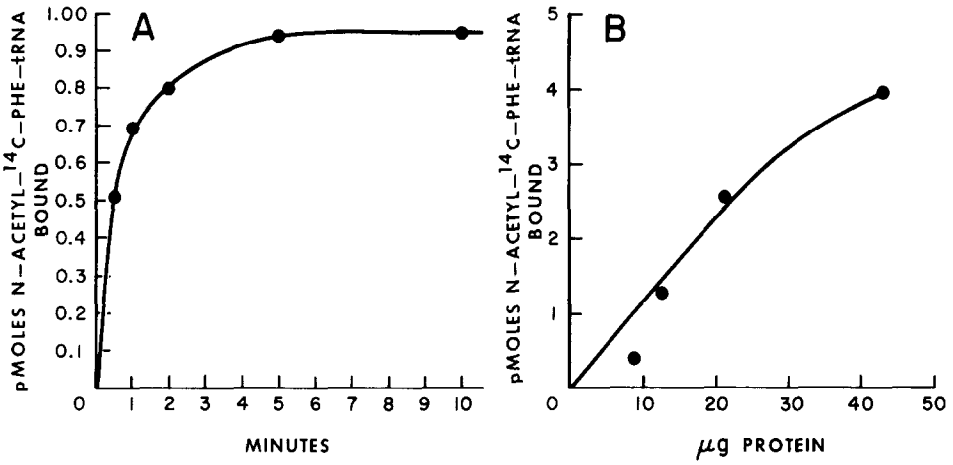

FIG. 1. Effect of time and protein concentration on the interaction of N-acetyl-^{14}C-Phe-tRNA with a soluble ribosomal protein. Fig. 1A: Each incubation contained 10 μg of ribosomal protein, and the reaction was carried out at 0^{o} for the time indicated. Fig. 1B: Each incubation was carried out for 5 min at 0^{o}. The details of the incubation and assay conditions are described in the text.

inactivated by heating at 55^{o} for 2 min in dilute salt, although in higher salt concentrations (1 M KCl) only 45% of the activity was destroyed by heating at 100^{o} for 10 min.

The inhibition of the AA-tRNA binding by poly U suggested that poly U may also interact with factors present in the ribosomal extract. As shown in Fig. 2, radioactive poly U was also retained by a nitrocellulose filter

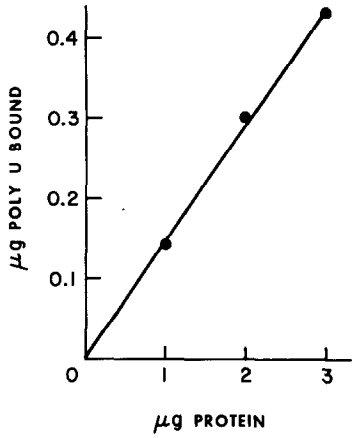

FIG. 2. Effect of protein concentration on the binding of poly U to a soluble ribosomal protein. Details of the incubation and assay conditions are described in the text.

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in the presence of the extract. This reaction occurred almost immediately at 0^{o}, and as shown in Fig. 2, the extent of reaction at 0^{o} was dependent on the amount of extract added. It is of interest to note that poly U binding activity was also observed in a 150,000 x g supernatant extract of E. coli. Smolarsky and Tal (16,18) have recently reported a poly U binding protein which could be removed from ribosomes by prolonged dialysis against 1 mM Tris-acetate buffer, pH 7.2. Brown and Doty (19) have also reported that a protein factor present in an initiation factor preparation binds the oligonucleotide AUG $(poly\ A)_{40}$, but whether these proteins are one and the same is not known.

Since it is now accepted that the enzymatic binding of AA-tRNA to ribosomes involves an AA-tRNA-Tu-GTP complex (8-10,20-22), the ribosomal extract was also examined for its ability to interact with this ternary complex. The complex was prepared with ^{14}C-phenylalanine (Phe) and γ-^{32}P-^{3}H-GTP, which made it possible to determine whether both the aminoacyl-tRNA and GTP moieties were involved in the reaction with the soluble extract. With intact ribosomes and poly U, the ternary complex is cleaved during the binding of the AA-tRNA to the ribosome-messenger complex (8-10). This reaction is conveniently

assayed using nitrocellulose filters since the Tu-GDP formed during this process as well as the ribosome bound AA-tRNA are retained by the filter. However, the unreacted ternary complex and any inorganic phosphate released are not bound to the filter.

Table II shows that the ribosomal extract also interacted with the ternary complex as evidenced by the retention of ^{14}C, ^{3}H, and ^{32}P by the filter. Further evidence that the appearance of both ^{3}H and ^{32}P on the filter is related to the formation of the AA-tRNA-Tu-GTP complex is shown in experiment 1. In these experiments (lines 2,3, and 4), the preparation of the complex was carried out as described in Methods, except that each of the components indicated was omitted from the incubation in which the complex was prepared. Thus, lines 2 and 4 show that there was no γ-^{32}P-^{3}H-GTP retained by the filter when the preparation of the complex was carried out in the absence of either Phe-tRNA or Tu. The finding that ^{14}C is found on the filter in the absence of GTP or Tu is in agreement with the previous results (Table I) which showed that the ribosomal extract is capable of binding Phe-tRNA.

An approximate ratio of 1:1:0.5-0.7 for ^{3}H:^{14}C:^{32}P was present on the filter after incubating the ternary complex with the ribosomal extract. The nature of the material retained on the filter has not yet been determined, but the presence of all three isotopes suggests that either the whole complex is bound, or that the complex has been split yielding AA-tRNA bound to the soluble ribosomal factor and Tu-GTP. The lower ^{32}P value relative to that of ^{3}H and ^{14}C also indicates that some hydrolysis of the GTP in the complex has occurred. Table II also shows that when poly U is added to the incubation containing the complex and ribosomal extract, there is an inhibition in the amount of all three isotopes found on the filter. Furthermore, it can be seen that the inhibition affected all three isotopes similarly since the isotopic ratio in the presence and absence of poly U remained approximately the same.

The results from these experiments show that when ribosomes are treated

TABLE II

Interaction of a Phe-tRNA-Tu-GTP Complex with a Ribosomal Soluble Factor

Experiment	Preparation of complex	Interaction of complex with soluble factor			
		Radioactivity bound to filter			
		^{3}H	^{14}C	^{32}P	^{3}H:^{14}C:^{32}P
		pmoles			
I	Complete	1.58	1.82	0.90	1.0:1.1:0.58
	-^{14}C-Phe-tRNA	0	-	0	
	-^{3}H-^{32}P-GTP	-	1.51	-	
	- Tu	0	1.58	0	
II	Complete	1.14	1.09	0.54	1.0:0.95:0.47
	"	0.45*	0.35*	0.21*	1.0:0.78:0.47
III	Complete	0.54	0.54	0.36	1.0:1.0:0.67
	"	0.23*	0.24*	0.16*	1.0:1.0:0.67

The preparation of the complex is described in the text. In experiment 1, where indicated, each of the components was omitted during the preparation of the complex. The complex once formed was incubated with the ribosome soluble factor as described in the text, and the interaction of the complex with the factor was determined by the retention of radioactivity on a nitrocellulose filter. The incubation mixtures in experiments I, II, and III contained 30 μg, 20 μg, and 26 μg, respectively of the ribosomal soluble factor.

*Poly U (5 μg) was added to the incubation mixture containing the complex and the ribosomal soluble factor.

with 1 M KCl and 2 mM Mg^{++}, proteins are released which are apparently soluble since they cannot be pelleted at 175,000 x g for 2 hrs. Other experiments have shown that all of the binding activities described here are in the supernatant fraction even after centrifugation of the extract for 16 hrs at 175,000 x g. In addition, preliminary evidence has been obtained which shows that the AA-tRNA and poly U binding factor(s) are not found in the void volume of the column when chromatographed on Sephadex G-100. It should be noted that the ribosomal pellet obtained after the high salt treatment is no longer able to bind N-acetyl-Phe-tRNA or Phe-tRNA (in the presence of poly U) either enzymatically or nonenzymatically.

The supernatant fraction has the ability to bind N-acetyl-Phe-tRNA, Phe-tRNA, and poly U as well as to interact with an AA-tRNA-Tu-GTP complex. Although a detailed RNA specificity has not been done, deacylated tRNA has also been shown to be bound by the extract which suggests that other high molecular weight RNA-like molecules might react with the extract. Since the proteins solubilized from the ribosomes were originally associated with ribosomal RNA and many are cationic in nature, it could be expected that some, if not all, of the ribosomal proteins would interact nonspecifically with polyanionic substances. However, as judged by the nitrocellulose filter assay, the reaction of the extract with the RNA species was not seen with the bulk of the proteins in the extract. On Sephadex G-100 chromatography the active material clearly separated from more than half of the protein in the extract. The experiments in progress will try to determine whether the observed interactions involve only one protein, and what the nature of the products are when the AA-tRNA-Tu-GTP complex reacts with the extract.

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