EVIDENCE FOR A GUANINE NUCLEOTIDE-AMINOACYL-RNA COMPLEX AS AN INTERMEDIATE IN THE ENZYMATIC TRANSFER OF AMINOACYL-RNA TO RIBOSOMES

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In recent investigations in this laboratory (1,2) two fractions required for polyuridylic acid-directed synthesis of polyphenylalanine were obtained from extracts of Escherichia coli W by chromatography on DEAE-Sephadex. One of the fractions, designated F-I, catalyzes the binding of aminoacyl-RNA to ribosomes in the presence of mRNA and GTP. This fraction has a high affinity for GTP and contains a GTPase that is stimulated by aminoacyl-RNA but not by stripped sRNA. Recently, Allende et al. (3) have shown that an enzyme fraction from E. coli B, which appears to be comparable to F-I, binds ^{3}H-GTP. In the present investigation ^{3}H-GTP was also found to interact with F-I to form a complex that is retained on a Millipore filter; however, if γ-^{32}P-GTP is used, very little radioactivity is retained by the filter. On further investigation the amount of ^{3}H-labeled compound bound to the protein was found to be decreased by the addition of aminoacyl-RNA but not by stripped sRNA nor by N-acetylaminoacyl-RNA. Preliminary data indicate that F-I catalyzes the formation of a guanine nucleotide-aminoacyl-RNA complex which may be the intermediate product formed in the "enzymatic" transfer of aminoacyl-RNA to ribosomes.

EXPERIMENTAL

Materials.--E. coli B sRNA was obtained from General Biochemicals. ^{14}C-Phenylalanyl-RNA prepared as described previously (2) was dissolved in 0.01 M potassium succinate, pH 5.6, and passed through a Sephadex G-25 column prepared in succinate buffer rather than acetate buffer. Washed ribosomes from E. coli W were prepared as previously described (2). N-Acetylphenylalanyl-RNA was

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prepared from phenylalanyl-RNA by the method of Haenni and Chapeville (4). ^{3}H-GTP was purchased from Schwarz BioResearch, Inc., and γ-^{32}P-GTP was purchased from International Chemical and Nuclear Corporation.

Preparation of F-I.--The 40-65 per cent ammonium sulfate fraction prepared from extracts of E. coli W was resolved into two fractions, F-I and F-II, by adsorption on a DEAE-Sephadex column and elution with a potassium chloride gradient as previously described (2). The flow rate of the column was decreased to 0.25 ml per minute. The column fractions catalyzing the transfer of phenylalanyl-RNA to ribosomes (F-I) were pooled, and diluted with 0.01 M Tris-HCl buffer, pH 7.5, containing 0.001 M dithiothreitol to lower the concentration of KCl to 0.15 M. The pooled, diluted fractions were then passed through a small DEAE-Sephadex column equilibrated in the same buffer, and the activity was eluted with buffer containing 0.3 M KCl.

Binding Assay.--The reaction mixture contained in a total volume of 0.5 ml: Tris-HCl buffer, 0.1 M, pH 7.7; dithiothreitol, 5 mM; NH_4Cl, 0.08 M; KCl, 0.08 M; $MgCl_2$, 12 mM; ^{3}H-GTP, 2 μM (specific activity 320 μc/μmole) and enzyme, as indicated. After 10 minutes of incubation at 0°, the reaction mixture was diluted 10-fold with 0.05 M Tris-HCl buffer, pH 7.7, containing 0.16 M NH_4Cl and 0.012 M $MgCl_2$, and the diluted reaction mixture was passed through a Millipore filter (0.45μ pore size). The filter was washed thoroughly with the same buffer, dried and counted in a Beckman scintillation counter.

RESULTS AND DISCUSSION

As shown in Fig. 1A there appears to be an interaction between protein present in F-I and ^{3}H-GTP, and the complex formed is retained by the Millipore filter. If, however, γ-^{32}P-GTP is incubated with F-I, the amount of ^{32}P-labeled material retained by the filter is about 10 per cent of that anticipated on the basis of the amount of ^{3}H-labeled complex retained by the filter. These results suggest that a guanine nucleotide-enzyme complex is formed with the loss of the γ-^{32}P moiety of the GTP. When phenylalanyl-RNA is added to the reaction mixture the amount of ^{3}H-labeled complex retained by the filter is greatly decreased (Fig. 1B). In separate experiments higher concentrations of phenylalanyl-RNA were found to decrease to an even greater extent the amount of ^{3}H-guanine nucleotide bound.

In Table I the effects of sRNA and aminoacyl-RNA on the amount of ^{3}H guanine nucleotide bound to F-I are shown. Only about a 10 per cent reduction in the amount of ^{3}H-labeled complex retained by the filter is observed when sRNA or N-acetylphenylalanyl-RNA is added to the reaction mixture, whereas more than a 70 per cent decrease is observed in the presence of either phenylalanyl-RNA or lysyl-RNA. When F-I is incubated with ^{3}H-GTP for

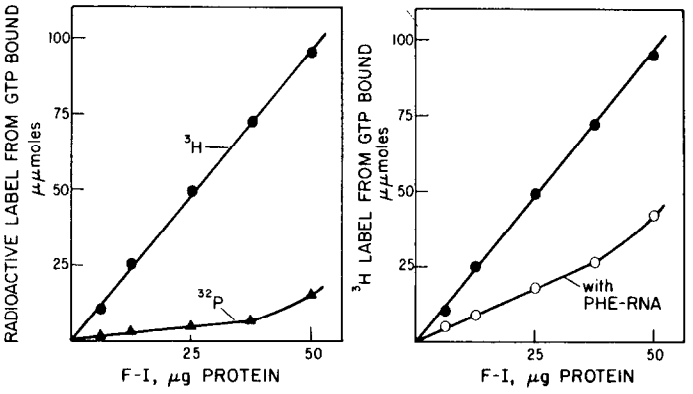

Figure 1. The interaction of ^{3}H-GTP and γ-^{32}P-GTP with F-I and the effect of phenylalanyl-RNA on the interaction of the ^{3}H-GTP. In A the reaction mixture described in Experimental Procedure contained F-I, 25 μg of protein, and either ^{3}H-GTP, 2 μM (●—●), or γ-^{32}P-GTP, 2 μM (▲—▲). In B the reaction mixture contained F-I, 25 μg of protein, 2 μM ^{3}H-GTP and when indicated, phenylalanyl-RNA, 0.1 mg of RNA charged with 72 μμmoles of phenylalanine.

TABLE I

The Effect of Aminoacyl-RNA on the Interaction of ^{3}H-GTP and F-I

	Radioactive Label Bound	
Supplements	^{3}H-GTP	^{14}C-Aminoacyl-RNA
	μμmoles	
None	47	-
sRNA	44	-
N-acetyl-^{14}C-Phe-RNA	42	< 0.1
^{14}C-Lys-RNA	12	< 0.1
^{14}C-Phe-RNA	13	< 0.1
^{14}C-Phe-RNA*	17	< 0.1
None*	63	-

The reaction mixture described in Experimental Procedure contained F-I, 25 μg of protein, and additional supplements as follows: sRNA, 0.1 mg; N-acetylphenylalanyl-RNA, 0.2 mg (72 μμmoles of phenylalanine); lysyl-RNA, 0.1 mg (82 μμmoles of lysine); phenylalanyl-RNA, 0.1 mg (70 μμmoles of phenylalanine).

* The reaction mixture was incubated for 10 minutes at 0°, the phenylalanyl-RNA added, and the incubation continued for an additional 10 minutes. The control was incubated for 20 minutes at 0°.

10 minutes, prior to the addition of the phenylalanyl-RNA, the same decrease in the ^{3}H-labeled complex retained by the filter is observed. The observed reduction in the ^{3}H-labeled complex by the addition of ^{14}C-aminoacyl-RNA is not paralleled by the appearance of a ^{14}C-labeled complex on the filter.

In other experiments no significant interaction of the ^{3}H-GTP with F-II or with F-I which had been heated at 55° for 4 minutes could be detected. ^{3}H-GDP was found to interact with F-I to the same extent as ^{3}H-GTP; however, the amount of ^{3}H-labeled complex formed from GDP and F-I was not diminished by the addition of aminoacyl-RNA.

Gel filtration of the incubation mixture containing F-I, ^{3}H-GTP and ^{14}C-phenylalanyl-RNA is shown in Fig. 2. Of the 280 μμmoles of the ^{14}C-phenylalanyl-RNA present in the incubation mixture, 255 μμmoles were recovered in fractions 8-12 and 247 μμmoles of the ^{3}H-label from the ^{3}H-GTP were recovered in these fractions. Calculations from the data indicate that fractions 9 and 10 contained 69 and 64 μμmoles of ^{3}H-labeled material and 70 and 67 μμmoles of the ^{14}C-labeled material, respectively, per 0.1 mg of sRNA. These results offer evidence for the formation of a guanine nucleotide-aminoacyl-RNA complex.

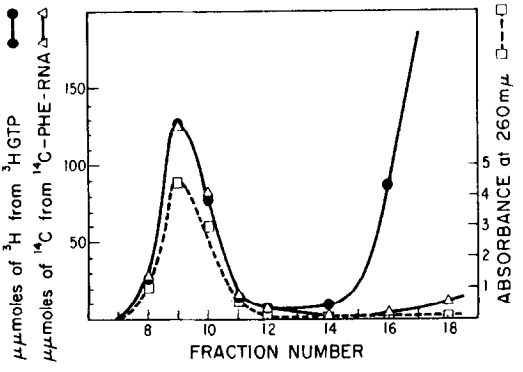

Figure 2. Gel filtration of the guanine nucleotide-phenylalanyl-RNA complex. The reaction mixture described in Experimental Procedure was increased to 1 ml and supplemented with F-I, 100 μg of protein, and 0.4 mg of RNA charged with 280 μμmoles of ^{14}C-phenylalanine. After 30 minutes of incubation at 0°, the reaction mixture was passed through a Millipore filter, and the filter was washed with 1 ml of 0.05 M Tris-HCl buffer, pH 7.7 containing 0.16 M NH_4Cl and 12 mM $MgCl_2$. The filtrate and wash were then placed on a Sephadex G-25 column (1 X 27 cm) equilibrated in the same buffer. The column was washed with buffer and fractions of 1 ml were collected.

TABLE II

The Binding of Phenylalanyl-RNA to Ribosomes

Supplements	Radioactive Label Bound		
	^{14}C-Phe-RNA	^{3}H-Guanine Nucleotide-^{14}C-Phe-RNA complex	
μμmoles	^{14}C	^{14}C	^{3}H
		μμmoles	
10	3.8*	4.0	3.0
20	4.0*	7.2	7.5
30	4.3*	9.1	10.1

The reaction mixture contained in a total volume of 0.5 ml: Tris-HCl buffer, 0.1 M, pH 7.7; dithiothreitol, 5 mM; NH_4Cl, 0.08 M; KCl, 0.08 M; $MgCl_2$, 12 mM; poly U, 10μg; washed ribosomes, 0.1 mg; and either ^{14}C-phenylalanyl-RNA or the ^{3}H-guanine nucleotide-^{14}C-phenylalanyl-RNA complex (Fractions 9 and 10 from the Sephadex G-25 column) as indicated. After 20 minutes of incubation at 25°, the reaction mixture was diluted with cold buffer, passed through a Millipore filter, and the filter washed, dried and counted as described above.

* The amount of phenylalanyl-RNA bound to ribosomes is increased to 8, 10, and 10.5 μμmoles, respectively, by the addition of F-I (6 μg protein) and GTP (2 μM) to the reaction mixture.

The ability of the complex to bind ribosomes in the presence of poly U was determined, and the results are shown in Table II. The amount of phenylalanyl-RNA bound to the ribosomes in the absence of enzyme and GTP ("non-enzymatic" binding) is approximately 4 μμmoles per 0.1 mg of ribosomes and does not increase appreciably when the concentration of phenylalanyl-RNA is increased from 10 μμmoles to 30 μμmoles. On the addition of F-I and GTP to the reaction mixture, the amount of phenylalanyl-RNA bound to the ribosomes increases approximately 2-fold as reported previously (1,2). With increasing concentrations of the guanine nucleotide-phenylalanyl-RNA complex, the amount of the phenylalanine moiety bound to the ribosomes reaches a level which is approximately twice the level obtained with phenylalanyl-RNA alone ("non-enzymatic" binding) and which is equivalent to the amount of phenylalanine bound to the ribosomes in the presence of phenylalanyl-RNA, enzyme and GTP. These data indicate that the complex is capable of binding the ribosomes in both the "non-enzymatic" and the "enzymatic" sites. It is of interest to note that the ^{3}H-guanine nucleotide portion of the complex is also bound to the ribosomes.

The results obtained in this investigation indicate that F-I reacts with GTP to form a guanine nucleotide-enzyme complex with the loss of the γ-phosphate moiety and that there is an interaction of the guanine nucleotide-enzyme complex with aminoacyl-RNA to form a guanine nucleotide-aminoacyl-RNA complex which is capable of binding ribosomes in both the "non-enzymatic" and the "enzymatic" sites. The nature of this intermediate and its role in peptide bond formation is being investigated.

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