The Composition of the Active Intermediate in the Transfer of Aminoacyl-RNA to Ribosomes

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It was first shown in this laboratory (1,2) that one of the fractions obtained from extracts of Escherichia coli W by chromatography on DEAE-Sephadex catalyzes the GTP-dependent binding of aminoacyl-RNA to E. coli ribosomes in a manner analogous to that previously reported for the rabbit reticulocyte system (3,4). Further investigation (5,6) indicated that this fraction interacts with GTP in the presence of aminoacyl-RNA to form a complex which serves as the active intermediate in the enzymatic transfer of aminoacyl-RNA to E. coli ribosomes. Similar findings have also been reported by other investigators (7-12). Evidence has been obtained recently (13,14) that two protein fractions, differing in heat lability, are required for the formation of a GTP-protein complex and for the GTP-dependent transfer of aminoacyl-RNA to ribosomes. In the present investigation, evidence is presented which demonstrates that the active intermediate for the transfer of aminoacyl-RNA to ribosomes is a complex composed of aminoacyl-RNA, GTP, and the heat labile transfer factor.

Experimental

Materials.--^{3}H-GTP was obtained from Schwarz BioResearch Inc., and γ-^{32}P-GTP was obtained from International Chemical and Nuclear Corporation. Ribosomes and ^{14}C-phenylalanyl-RNA were prepared as previously described (2,5). F-IA, F-IB, and F-II were obtained by DEAE-Sephadex chromatography as previously described (6) and were stored at -90°.

Assays.--Transfer assay.--The reaction mixture contained in a total volume of 0.5 ml: Tris-HCl buffer, 0.05 M, pH 7.7; dithiothreitol, 5 mM; NH_4Cl, 0.08 M; KCl, 0.08 M; $MgCl_2$, 0.01 M (Buffer A); ribosomes, 0.1 mg; poly U, 10 µg; tRNA, 50 µg charged with 32 µµmoles of ^{14}C-phenylalanine, and other supplements, as

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indicated. After 10 minutes of incubation at 25°, the ^{14}C-phenylalanyl-RNA bound to ribosomes was determined as previously described (2).

Polymerization assay.--The reaction mixture described above was modified to contain: 50 mμmoles of GTP; 100 μg of tRNA charged with 65 μμmoles of ^{14}C-phenylalanine; fraction F-II, 2 μg of protein, and other supplements, as indicated. After 20 minutes of incubation at 25°, the amount of hot TCA insoluble material was determined as previously described (2).

Complex formation.--Duplicate reaction mixtures were prepared containing Buffer A, 1 mμmole of ^{3}H-GTP, and enzyme fractions in a total volume of 0.5 ml To one of the reaction mixtures, 0.2 mg of tRNA charged with 120 μμmoles of phenylalanine was added. After 10 minutes of incubation at 0°, the reaction mixtures were diluted with buffer and passed through pre-soaked Millipore filters. The filters were washed and counted as previously described (5). The amount of phenylalanyl-RNA-GTP-complex formed was calculated from the decrease in the amount of ^{3}H-labeled complex retained by the filter in the presence of phenylalanyl-RNA.

Results and Discussion

The data presented in Table I indicate that two factors, differing in heat sensitivity, are required for the formation of an aminoacyl-RNA-GTP complex that is not retained by a Millipore filter. The factor present primarily in F-IA is stable to heating at 50° for 10 minutes but is inactivated by heating at 60° for 10 minutes. The factor present primarily in F-IB is inactivated by heating at 50° for 10 minutes. In the experiment shown in Table I, the amount of phenylalanyl-RNA-GTP complex formed was estimated from the decrease in the amount of complex retained by a Millipore filter due to the addition of phenylalanyl-RNA to the reaction mixture. In separate experiments the amount of phenylalanyl-RNA-GTP complex in the Millipore filtrat was measured by chromatography on Sephadex G-25, and comparable values were obtained.

Since two factors, one stable and one labile at 50°, are required, in addition to GTP and aminoacyl-RNA, for the formation of the active intermediate involved in the enzymatic binding of aminoacyl-RNA to ribosomes, it was of considerable interest to determine the composition of the active intermediate. As shown in Fig. 1a, the Millipore filtrate obtained from the interaction of F-IA and F-IB with GTP and phenylalanyl-RNA is as active as the unfiltered reaction mixture in transferring phenylalanyl-RNA to ribosomes. In contrast, the filtered and unfiltered reaction mixtures do not exhibit equal activity in forming polyphenylalanine in the presence of F-II, additional phenylalanyl-RNA, and GTP (Fig. 1b). The filtrate is approximately one-fourth as active

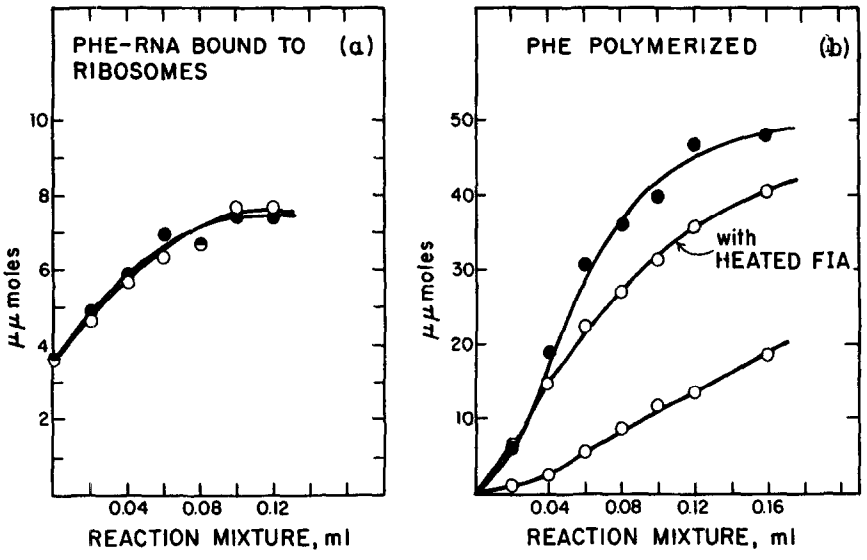

Fig. 1. The activities of filtered and unfiltered reaction mixtures. The reaction mixture contained in a volume of 4 ml: Buffer A; F-IA, 100 μg of protein; F-IB, 400 μg of protein; GTP, 8 mμmoles; and 1 mg of tRNA charged with 640 μμmoles of ^{14}C-phenylalanine. After 10 minutes of incubation at 0°, 2 ml were passed through a double layer of Millipore filters and the filtrate collected. The activities of the unfiltered (●—●) and filtered (O—O) reaction mixtures in the transfer and polymerization assays were determined as described in Methods. Heated F-IA, 2.5 μg of protein, was added when indicated.

as the unfiltered reaction mixture. The addition of the more heat stable factor (F-IA heated at 50° for 10 minutes) almost completely restores the activity of the filtrate. In separate experiments, only a slight enhancement of the activity of the filtrate was obtained by the addition of the heat labile factor (F-IB). The ability of the filtrate to transfer phenylalanyl-RNA to ribosomes or to form polyphenylalanine, either in the absence or presence of heated F-IA, is destroyed by heating the filtrate at 50° for 10 minutes. These results indicate that the heat labile transfer factor is a component of the complex present in the Millipore filtrate, and that the heat stable factor is necessary for the formation of additional complex.

Chromatography of the Millipore filtrate on Sephadex G-100 (Fig. 2a) demonstrates that the phenylalanyl-RNA is associated with the GTP complex, although considerable dissociation of the phenylalanyl-RNA portion of the complex occurs during filtration. Previously only indirect evidence for the association of the aminoacyl-RNA with the active GTP complex had been obtained (5,6,9,10,15). As shown in Fig. 2b, polyphenylalanine synthesis is obtained

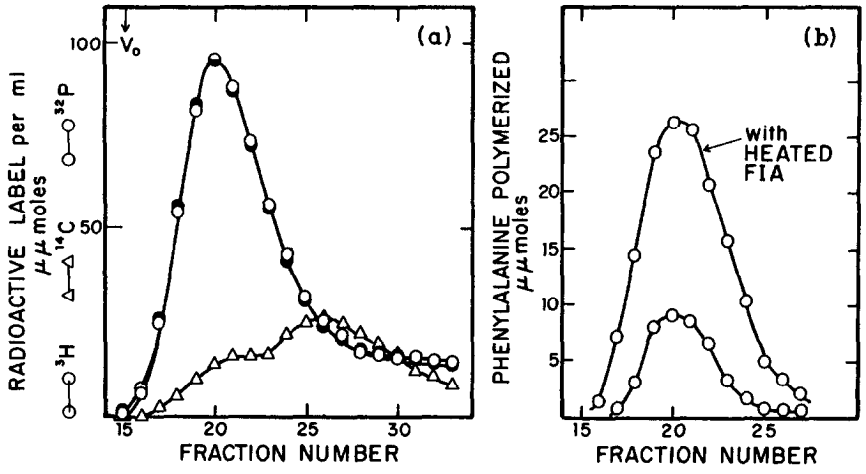

Fig. 2. Chromatography of Millipore filtrate on Sephadex G-100. The reaction mixture contained in a volume of 1 ml: Buffer A; F-IA, 100 μg of protein; F-IB, 400 μg of protein; ^{3}H-GTP, 1 mμmole, 200,000 cpm; γ-^{32}P-GTP, 1 mμmole, 300,000 cpm; and 0.8 mg tRNA charged with 500 μμmoles of ^{14}C-phenylalanine. After 10 minutes of incubation at 0°, the reaction mixture was passed through a triple layer of Millipore filters, and the filters were washed with 0.5 ml of Buffer A. The filtrate and wash were applied to a Sephadex G-100 column (1.5 x 27 cm) equilibrated in Buffer A. The column was washed with Buffer A and 1 ml fractions were collected. In a) the radioactivity present in a 0.2 ml aliquot of each fraction was measured in a Beckman scintillation counter, and the total amount of ^{3}H, ^{14}C and ^{32}P labeled material present in each 1 ml fraction was calculated. In b) the activity of a 0.2 ml aliquot of each fraction was measured in the polymerization assay described in Methods. Heated F-IA, 5 μg of protein, was added when indicated.

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with fractions 18-24 and is proportional to the amount of GTP complex present in each fraction. As in the case of the Millipore filtrate, the activity of fractions 18-24 in the polymerization assay is enhanced by the addition of heated F-IA. The ability of these fractions to transfer phenylalanyl-RNA to ribosomes or to form polyphenylalanine, either in the absence or presence of heated fraction F-IA, is destroyed by heating at 50° for 10 minutes. The total amount of protein present in fractions 18-24 determined by the Folin-Lowry method is approximately 50 μg.

Additional evidence that the heat labile transfer factor is the protein component of the active intermediate is obtained by chromatography of the Millipore filtrate on Sephadex G-100 equilibrated with buffer containing no Mg^{++}. Under these conditions, not only the phenylalanyl-RNA but also the GTP dissociates from the complex. Fractions 18-24 obtained in this manner exhibit very little activity when assayed alone but are as active as fractions 18-24 in Fig. 2b when assayed in the presence of heated F-IA.

Table I

Evidence for the Involvement of Two Factors in the Formation of an Aminoacyl-RNA-GTP Complex

Fraction	Phenylalanyl-RNA-GTP Complex Formed
	μμmoles
F-IA	8
Heated F-IA	3
F-IB	16
Heated F-IB	3
F-IA + F-IB	129
Heated F-IA + F-IB	123
F-IA + Heated F-IB	20
Heated F-IA + Heated F-IB	17

Complex formation was measured as described in Methods. The reaction mixtures were supplemented with F-IA, 25 μg protein and F-IB, 100 μg protein, as indicated. F-IA and F-IB were heated at 50° for 10 minutes.

The results of this investigation demonstrate that: 1) Although two transfer factors, one stable and one labile at 50°, are required for the formation of the aminoacyl-RNA-GTP-protein complex that is the active intermediate in the transfer of aminoacyl-RNA to ribosomes, only the heat labile transfer factor is a component of the active intermediate recovered in the Millipore filtrate. 2) Polymerization is limited by the amount of active intermediate present in the Millipore filtrate, and the more heat stable transfer factor is required for the formation of additional intermediate.

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