IDENTIFICATION OF THE TWO ^{3}H-GTP BINDING PROTEINS IN *E. coli* SUPERNATANT AS Tu-Ts AND Tu FACTORS

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Summary Of the two ^{3}H-GTP-binding components separated on DEAE-Sephadex column chromatography of an *E. coli* supernatant fraction, the one was identified as Tu-Ts complex and the other as Tu. This assignment was based on the observation that the former rapidly binds with ^{3}H-GDP whereas the latter binds with it only slowly. This was further confirmed by elution pattern of the two components from Sephadex G-100 column. Molecular weight of Tu, Ts and Tu-Ts was estimated to be approximately 5×10^4, 5×10^4, and 1×10^5, respectively.

Introduction

Two complementary factors, T and G(1), are required for the elongation of peptide bonds on ribosomes in a cell-free system of *E. coli*. Factor T has been further divided into Tu and Ts components(2) and their properties and mode of action have extensively been studied in recent years(for recent review see refs. 3 and 4). In the presence of GDP or GTP, Tu-Ts complex has a tendency to dissociate into Tu and Ts which in turn reassoiate to form Tu-Ts complex in the absence of the guanine nucleotide(5, 6). Recently Ts was shown to catalyze the exchange of free GDP with Tu-GDP according to the equations below(7).

$$\text{Tu-GDP} + \text{Ts} \rightleftharpoons \text{Tu-Ts} + \text{GDP} \qquad (1)$$

$$\text{Tu-Ts} + {}^3\text{H-GDP} \rightleftharpoons \text{Tu-}{}^3\text{H-GDP} + \text{Ts} \qquad (2)$$

$$\text{Sum: Tu-GDP} + {}^3\text{H-GDP} \underset{(\text{Ts})}{\rightleftharpoons} \text{Tu-}{}^3\text{H-GDP} + \text{GDP} \qquad (3)$$

In the purification of the elongation factors from the *E. coli* supernatant fraction, Nishizuka and Lipmann(1) separated three peaks on DEAE-Sephadex column of which they defined the first and the third ones as T and G factors, respectively. The second peak was referred to as G-T complex because each fraction in this peak by itself was active in poly U directed polyphenylalanine synthesis. On the other hand Ertel *et al.* (8) found two peaks of ^{3}H-GTP binding activity in a similar column chromatography and suggested that the main components of the first(peak 1) and the second(peak 2) peaks are Ts and Tu, respectively, because ^{3}H-GTP binding by peak 2 protein was stimulated by peak 1 protein. It seems dif-

ficult, however, to explain a substantial ^{3}H-GTP binding activity in peak I merely by contamination with Tu.

We have also obtained a double peak(peaks I and II) of T activity on DEAE-Sephadex column(9) probably corresponding to peaks I and 2 of Ertel et al. (8). The evidence to be reported in this communication indicates that peaks I and II are Tu-Ts and Tu, respectively.

Materials and Methods

Tu and Ts activities were measured by binding with ^{3}H-GDP using the nitrocellulose membrane procedure(10). The reaction mixture contained in a total volume of 0.04 ml, ^{3}H-GDP (specific activity, 1 mC/μmole), 2.5 μM; Tris-HCl buffer(pH 7.5), 0.05 M; $MgCl_2$, 0.01 M; NH_4Cl, 0.15 M: 2-mercaptoethanol, 0.01 M: and Tu and Ts factors as indicated, unless otherwise specified. The reaction was started by addition of ^{3}H-GDP and the reaction mixture was incubated at 0°C for various periods as indicated. It was stopped by addition of 2 ml of a cold solution containing Tris-HCl buffer(pH 7.5), 0.01 M; and $MgCl_2$, 0.01 M and poured onto a nitrocellulose membrane filter(0.45 μ pore size). The filter was washed three times with 2 ml of the same solution, dried and counted in a liquid scintillation spectrometer. T factor(peaks I and II of DEAE-Sephadex column) from E. coli Q13 was prepared as described previously(9). Factor Ts was prepared by rechromatography of peak I protein on DEAE-Sephadex A-50 column. A fraction eluted at 0.14 M KCl was concentrated in a collodion bag under a reduced pressure and stored at -80°C. This preparation was practically free of Tu and showed no ability to bind ^{3}H-GDP unless supplemented with Tu. Units of Tu and Ts activity defined by Weissbach and his collaborators(11) are used in this communication.

Results and Discussion

As shown in Fig. 1A, when factors T and G were separated by DEAE-Sephadex column chromatography, T activity which was assayed by binding with ^{3}H-GTP using the Millipore membrane procedure appeared as two peaks(9). Both peaks I and II are complementary to G in poly U directed polyphenylalanine synthesis(Fig. 1B). When the polymerization of ^{14}C-phenylalanyl-tRNA was followed with each fraction alone, there was a peak between second T and G peak probably due to the presence of both T and G activities in the same fraction. This probably corresponds to G-T complex of Nishizuka and Lipmann(1).

In order to characterize the nature of the T activities present in peaks I and II, the ^{3}H-GDP binding activity of these fractions either alone or in combination was measured after incubation for 1 or 20 min at 0°C. The results in Table I shows that binding of ^{3}H-GDP to peak I protein was almost completed in 1 min, whereas the binding to peak II protein took place more slowly. Moreover the rate of ^{3}H-GDP binding to peak II protein was markedly

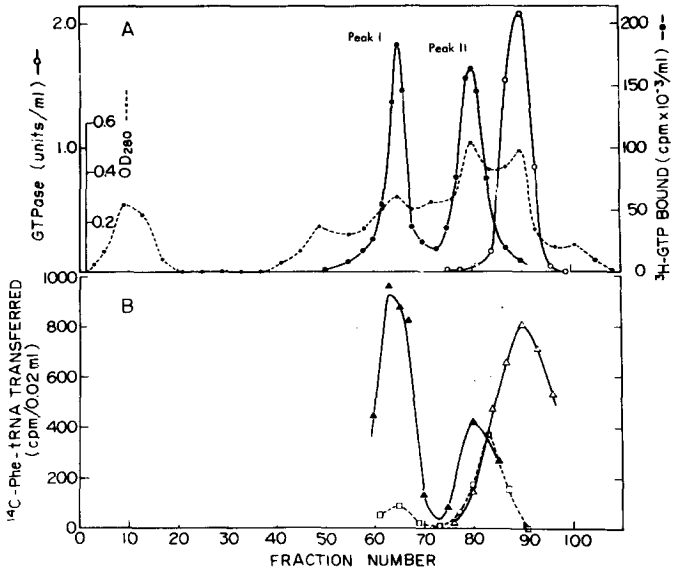

Fig. 1: DEAE-Sephadex A-50 column chromatography(9). The $(NH_4)_2SO_4$ fractions were applied to the DEAE-Sephadex A-50 column(1.5 x 80 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 7.5) containing 0.15 M KCl and 5 mM 2-mercaptoethanol and eluted in a 1200 ml linear gradient between 0.2-0.4 M KCl. Ten-ml fractions were collected.
A: ^{3}H-GTP binding activity (—●—) was measured under the standard conditions except that ^{3}H-GDP was replaced by ^{3}H-GTP, 6.25 μM(specific activity, 800 μC/μmole). The reaction mixture was incubated for 20 min. G factor was assayed by ribosome-dependent GTPase activity as described elsewhere(9).
B: Polymerization of ^{14}C-Phe-tRNA(specific activity, 300 μC/μmole phenylalanine) was assayed as described elsewhere(9). Twenty μl of each fraction was assayed either alone(--□--) or in the presence of 20 μl of fraction 90(—▲—) or in the presence of 40 μl of fraction 65 (—△—).

Table I

^{3}H-GDP binding with peak I and peak II proteins

	Time of incubation(min)	pmoles ^{3}H-GDP bound
Peak I	1	2.5
	20	2.8
Peak II	1	8.3
	20	30.9
Peak I + Peak II	1	39.5

^{3}H-GDP binding reaction was carried out under the standard conditions. 25.8 μg of peak I protein and 43.6 μg of peak II protein were used either alone or in combination in each experiment.

accelerated by peak I protein. These findings suggest that peak I contains both Tu and Ts activities while peak II contains only Tu with a limiting amount of Ts.

The nature of these peaks was further characterized by gel filtration on a Sephadex G-100 column. Chromatography of peak II protein on Sephadex G-100 gave an elution profile shown in Fig. 2A. Binding with ^{3}H-GDP was measured with each fraction in the presence and absence of added Ts. The binding was very low unless each fraction was supplemented with Ts indicating that the peak fractions have a characteristic of Tu-GDP. Fig. 2B shows an elution profile of Ts purified as described in "Materials and Methods" and chromatographed on the same column as above. Activity of Ts emerged almost at the same effluent volume as Tu, in accordance with Miller and Weissbach(5). When peak I protein was chromatographed on Sephadex G-100 column, both Tu and Ts activities were highest in fraction 22 and showed a similar elution profile(Fig. 2C). This finding together with the fact that

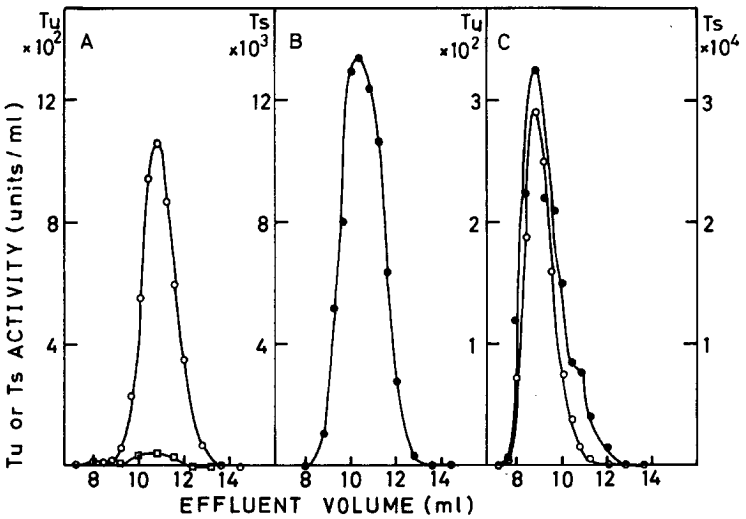

Fig. 2: Chromatography of peak II, Ts and peak I protein on Sephadex G-100. A Sephadex G-100 column(0.9 x 30 cm) was equilibrated with a solution containing Tris-HCl buffer(pH 7.5), 0.01 M; $MgCl_2$, 0.01 M; NH_4Cl, 0.01 M and 2-mercaptoethanol, 5 mM. The flow rate was adjusted to 4.5 ml/hr, and 0.4 ml fractions were collected. The void volume of the column was 7.4 ml. Each protein(in 0.1 ml) was applied to the column and eluted with the same buffer. A, 22 mg of peak II protein; B, 0.2 mg of Ts; and C, 15 mg of peak I protein were chromatographed. Tu activity of each fraction was measured in the presence of Ts(250 units). The reaction was carried out for 20 minutes at 0°C. Ts activity was measured in the presence of Tu(27 units) and the reaction was stopped at 1 min after addition of ^{3}H-GDP. Less than 60 % of added Tu was complexed within this period. In A, ^{3}H-GDP binding in the absence of Ts was also measured after incubation for 1 min at 0°C.

—o— Tu activity in the presence of Ts
—●— Ts activity in the presence of Tu
—□— ^{3}H-GDP binding in 1 min in the absence of Ts

the activities were eluted earlier than separated Tu or Ts preparation suggests that peak I protein mainly contained Tu-Ts complex. Binding of the peak fraction with ^{3}H-GDP in the absence of added Ts was really rapid and completed within I min.

The identity of peak I with Tu-Ts complex was further confirmed by the following experiment in which the complex was resolved into Tu-GDP and Ts by extensive dialysis against 10^{-5}M GDP and chromatographed on DEAE-Sephadex in the presence of 10^{-5}M GDP. This time, the peak of Tu-Ts at O.25M KCl was disappeared, and instead, two complementary components were eluted at O.14M and O.28M KCl, the concentrations at which Ts and Tu are respectively to be eluted. Thus, it is clear that peak I protein is composed of Tu-Ts complex which is resolved into Ts and Tu-GDP in the presence of excess GDP. The approximate molecular weights were calculated from the results of Sephadex G-100 chromatography in Fig.2 with cytochrome c, hemoglobin and catalase as standards. They were estimated to be 5×10^{4}, 5×10^{4}, and 1×10^{5} for Tu, Ts and Tu-Ts, respectively. One to one stoichiometry of Tu and Ts in Tu-Ts complex was thus demonstrated.

Kinetics of ^{3}H-GDP binding reaction was studied using Tu purified by Sephadex G-100. The time course of ^{3}H-GDP binding reaction with and without Ts is shown in Fig. 3. In the presence of Ts the reaction was completed within I min, whereas ^{3}H-GDP binding increased

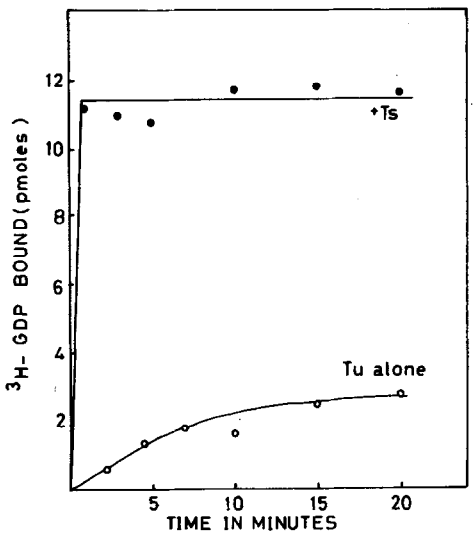

Fig. 3: Binding of Tu with ^{3}H-GDP in the presence and absence of Ts. Tu(II.5 units) purified by Sephadex G-100 was incubated with or without Ts(250 units) under the standard conditions. The reaction was stopped at the time indicated and the binding was measured as described in the text.
—●— ^{3}H-GDP binding in the presence of Ts.
—○— ^{3}H-GDP binding in the absence of Ts.

slowly for about 15 min in its absence and reached a plateau. This slow ^{3}H-GDP binding does not seem to be due to contamination with Ts because the amount of bound ^{3}H-GDP in the absence of added Ts was far less than the total binding capacity of Tu observed in its presence, varying from 25 to 50 % of the total depending on the preparation of Tu used. It seems probable that a mixture of Tu and/or Tu-GTP and Tu-GDP was obtained in the present purification. The amount of bound ^{3}H-GDP without added Ts probably represents the amount of Tu and Tu-GTP in the Tu preparation. Fig. 4 shows that ^{3}H-GDP once complexed with Tu could not be diluted out by cold GDP in the absence of Ts. Rapid exchange with cold GDP occurred only when Ts was added to the incubation mixture. These properties are consistent with those reported recently from other laboratories(5,6). Gradual exchange between free and bound nucleotides is also seen in Fig. 4. Whether this is due to a trace contamination with Ts or to that Tu-GDP by itself is capable of slow exchange is open at present.

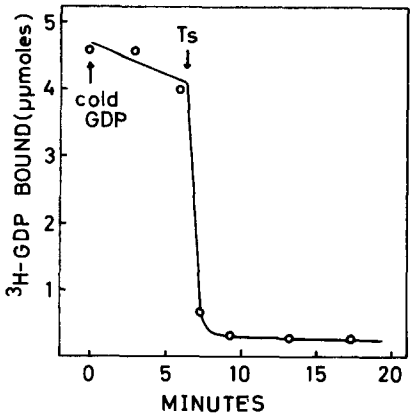

Fig. 4: Exchange of GDP bound to Tu with unbound GDP. The standard reaction mixture was scaled up to 0.2 ml. Tu(87 units) was preincubated in the presence of 2.5 μM ^{3}H-GDP for 30 min at 0°C and then 10 μl of cold GDP was added to give a final concentration of 0.45 mM. Incubation was continued at 0°C and 30 μl aliquots were transferred to 2 ml of a solution containing 10 mM Tris-HCl(pH 7.5) and 10 mM Mg Cl_2 at times indicated. Nitrocellulose filter assay was carried out as described under Materials and Methods. Ts(1,000 units in 5 μl) was added at 6.5 min after the addition of cold GDP.

References

1. Y. Nishizuka and F. Lipmann, Proc. Natl. Acad. Sci., U. S., 55, 212(1966).
2. J. Lucas-Lenard and F. Lipmann, Proc. Natl. Acad. Sci., U. S., 55,1562(1966).
3. F. Lipmann, Science, 164, 1024(1969).
4. P. Lengyel and D. Söll, Bact. Rev., 33, 264(1969).

5. D. L. Miller and H. Weissbach, Arch. Biochem. Biophys., 132, 146(1969).
6. J. Waterson, G. Beaud, P. Lengyel, Nature, 227, 34(1970).
7. H. Weissbach, D. L. Miller and J. Hachmann, Arch. Biochem. Biophys., 137, 262(1970).
8. R. Ertel, N. Brot, B. Redfield, J. E. Allende and H. Weissbach, Proc. Natl. Acad. Sci., U. S., 59, 861(1968).
9. Y. Kaziro, N. Inoue, Y. Kuriki, K. Mizumoto, M. Tanaka and M. Kawakita, Cold Spring Harbor Symp. Quant. Biol., 34, 385(1969).
10. J. E. Allende, N. W. Seeds, T. W. Conway and H. Weissbach, Proc. Natl. Acad. Sci., U. S., 58, 1566(1967).
11. R. Ertel, B. Redfield, N. Brot and H. Weissbach, Arch. Biochem. Biophys., 128, 331 (1968).