CRYSTALLINE TRANSFER FACTORS FROM ESCHERICHIA COLI

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The transfer of the aminoacids from the aminoacyl RNA donor to a ribosome bound polypeptide requires in the in vitro systems from different sources the presence of at least two soluble complementary enzymatic factors (1,2,3,4,5,6,7). The mechanism of action of these enzymes - one of which was shown to be coincident with a ribosome-dependent GTPase (8,9) - as well as their physico-chemical properties are still largely unknown. The present communication describes the purification and crystallization of the transfer factors from E. coli and some of their properties. In the last few years, several authors have reported the partial purification of these enzymes from E. coli (10), yeast (11) and liver (4, 12).

METHODS

E. coli, strain A19 or $BT2^r$, where grown in a medium containing 0.8% Bacto Nutrient Broth, Difco Lab., and 0.5% glucose.

Enzymatic activities were determined by measuring the poly-U directed phenylalanine polymerization and the GTP hydrolysis. Assay for polymerization - One ml of the reaction mixture contained: 80 µmoles Tris-HCl, pH 7.8; 4 µmoles $(NH_4)_2SO_4$; 18 µmoles KCl; 10 µmoles $MgCl_2$; 12 µmoles 2-mercaptoethanol; 40 µg poly-U; 550 µg ^{3}H-Phe-tRNA (3 mc/µmole); 1 mg ribosomes; 270 mµmoles GTP; 25 µmoles PEP; 1.5 µg pyruvate kinase; 1 µg of crystalline GTPase (G-I); and the enzyme fractions. The volume of a single assay was either 75 or 150 µl. After the induction time, when the reaction was proceeding linearly, several aliquots for each sample were withdrawn at 5 min intervals. The aliquots were

placed on GF/A filters (Whatman) which were immediately dipped in cold 10% TCA. After 15 min in 5% TCA at 95°, the filters were washed, dried and counted (13). Assay for GTPase - One ml of the reaction mixture contained 80 µmoles Tris-HCl, pH 7.8; 70 µmoles NH_4Cl; 10 µmoles $MgCl_2$; 12 µmoles 2-mercaptoethanol; 1 mg ribosomes; 550 mµmoles γ-^{32}P-GTP (0.1 - 0.5 µc/µmole); and the enzyme. The volume for each sample was either 75 or 150 µl. The reaction was stopped after 10 min by adding an equal volume of 1 M $HClO_4$. The liberated $^{32}P_i$ was extracted as phosphomolybdate complex in isopropylacetate (14). An aliquot of the organic phase was placed on GF/A filters, dried, and the radioactivity measured.

Protein concentrations were measured according to Lowry et al. (15). The ribosomes were 5 x washed in 0.5 M NH_4Cl (16). ^{3}H-Phe-tRNA was 1.5 - 2.5% charged (17). γ-^{32}P-GTP (1 mc/ mole) was synthetized from GDP and $^{32}P_i$ by spinach chloroplasts (8). Preparative polyacrylamide gel electrophoresis (Buchler Instruments, N.J.) was run in a 7.5% small pore gel using a Tris-glycine buffer system, pH 9.5 (18). Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer model 3003. Analytical ultracentrifugation was carried out with a Spinco model E.

RESULTS

Step I - The cells, 50 g wet weight, harvested in early log phase (A_{500} = 0.7), were washed, centrifuged, disrupted by grinding with alumina, and extracted at 2° (17). After treatment with DNAase (2 µg/ml extract) for 10 min, the extract was centrifuged twice at 30,000 x g for 30 min and the precipitate discarded. Step II - The supernatant was centrifuged at 140,000 x g for 2 hours. To each 100 ml of the clarified solution, 4 ml of neutralized 2% protamine sulfate solution were added. The turbid suspension was centrifuged at 30,000 x g and the precipitate discarded. Except were noted, all steps were carried out at 2° and in the presence of 7 mM 2-mercaptoethanol. Step III - The protein solution was fractionated with a saturated $(NH_4)_2SO_4$ solution between 40 and 65% saturation. The precipitate collected by centrifugation was

dissolved in Tris-HCl, pH 7.8, and dialyzed against the same buffer. Step IV - The pH of the solution was adjusted to 6.7. 2-Isopropanol was added to a concentration of 10% (v/v) in the presence of 50 mM $MgCl_2$. The temperature was lowered to -5° and the alcohol concentration increased to 22%. The precipitate collected by centrifugation was dissolved in 50 mM Tris-HCl, pH 7.8, and dialyzed against the same buffer. Step V - The protein solution was passed through a Sephadex G-150 column (5x100 cm) equilibrated with 50 mM Tris-HCl, pH 7.8, containing 1 mM dithiothreitol. After this stage, 1 mM dithiothreitol was present in all following steps, including crystallization. The active fractions were pooled, concentrated with Aquacide I (Calbiochem), and dialyzed against 10 mM phosphate buffer, pH 7.1. Step VI - The protein solution was applied to an hydroxyapatite column (6 x 4 cm) equilibrated with 10 mM phosphate buffer, pH 7.1. The column was washed with the same buffer and, thereafter, the protein was eluted at three different phosphate concentrations. At 30 mM, a transfer factor (G-I) showing GTPase activity was separated; at 60 mM, both GTPase and polymerization activity were associated (G-II+T-II); at 90 mM, a polymerization factor (T-I) without GTPase activity was isolated. Similarly, three separate active protein peaks were obtained using a shallow linear phosphate gradient from 10 to 100 mM. The three hydroxyapatite fractions were each purified in the following similar manner. Step VII - Each fraction was concentrated with Aquacide I. Saturated $(NH_4)_2SO_4$ solution was added to 40% saturation. The precipitate was centrifuged and discarded. The clear supernatant was dialyzed against 60 mM Tris-HCl, pH 6.7, and applied in 5% sucrose on the concentrating gel of a 5 cm long polyacrylamide gel column. The enzymes were eluted after 10 - 12 h at a current of 40 mA and 120 V. The active pools were concentrated to 3 - 4 ml with Aquacide I and dialyzed against Tris-HCl, pH 7.8. In the case of G-II and T-II, their activities were still slightly overlapping. Step VIII (Crystallization) - G-I: The clear solution was dialyzed against 40% saturated $(NH_4)_2SO_4$ in 50 mM Tris-HCl, pH 7.8. The $(NH_4)_2SO_4$ concentration was increased to 43 - 45% saturation until a faint turbidity appeared. Within a few hours

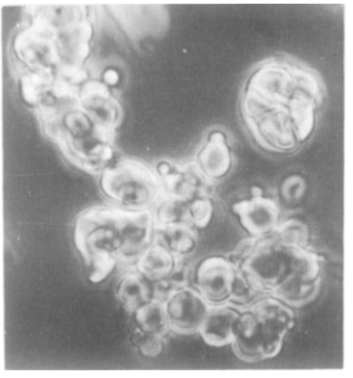

Fig. 1. Crystals of G-I, approx. 900 x.

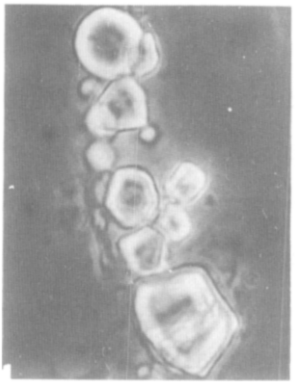

Fig. 2. Crystals of T-I, approx. 900 x.

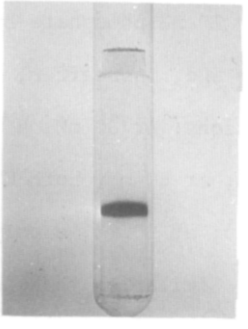

Fig. 3. Disc gel electrophoresis of crystalline G-I after 310 min at a current of 5 mA per cm^2. Protein amount: 70 µg.

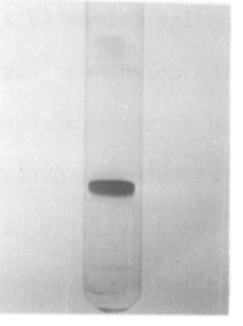

Fig. 4. Disc gel electrophoresis of crystalline T-I. Same running conditions as in Fig. 3. In the lower part of the gel column, the front mark is visible. Protein amount: 75 µg.

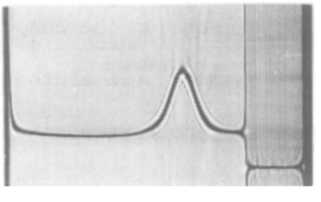

Fig. 5. Ultracentrifuge pattern of crystalline G-I in 50 mM Tris-HCl, pH 7.8, containing 100 mM KCl, 7 mM 2-mercaptoethanol and 1 mM dithiothretol after 49 min at 56.000 rpm. Protein concentration: 9 mg/ml.

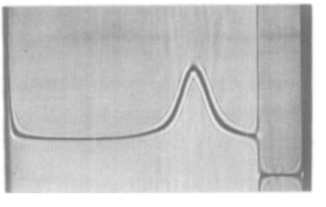

Fig. 6. Ultracentrifuge pattern of crystalline T-I after 56 min at 56.000 rpm. Ionic environment as in Fig. 5. Protein concentration: 9.5 mg/ml.

crystallization occurred. The $(NH_4)_2SO_4$ concentration was increased during a period of 1 - 2 days to 47 - 49% saturation at which time crystallization was complete. The polygonal or round crystalline plates formed characteristic crops (Fig.1). G-II: The same procedure as for G-I was applied. The crystalls obtained looked similar to those of G-I. T-II: The solution was dialyzed against 50% saturated $(NH_4)_2SO_4$ in Tris-HCl, pH 7.8. The precipitate which included the residual GTPase was centrifuged and added to G-II. The $(NH_4)_2SO_4$ concentration was increased to 54% saturation. The precipitate formed was centrifuged and discarded. Increase in the concentration of $(NH_4)_2SO_4$ caused appearance of a faint turbidity at about 58% saturation. Crystallization was complete at 64% saturation. The crystals occurred as typical rhomboids. T-I: The same procedure as for T-II was applied. The crystals appeared as polygonal plates (Fig.2).

The method as described here is reproducible and has been used for 12 different preparations. To obtain extracts of high activity, it is essential to grow the cells as reported. Suspensions of the crystalline enzymes stored in ammonium sulphate with 1 mM dithiothreitol and 7 mM 2-mercaptoethanol are stable for at least several weeks at 2°. In 50 mM Tris-HCl, pH 7.8, containing 1 mM dithiothreitol and 7 mM 2-mercaptoethanol, they retain activity for several days at 2°. The $A_{280} : A_{260}$ ratio is 1.75 for G-I and G-II, while for T-I and T-II it is 1.70. On analytical disc gel electrophoresis, all isolated fractions migrate as single components (Fig.3 and 4, G-II and T-II are not illustrated). Ultracentrifugation of G-I and T-I shows single symmetrical peaks (Fig.5 and 6) with $s^{\circ}_{20,w} = 5.3S$ and 5.1S, respectively. Tables I and II summarize the results of a typical preparation of G-I and T-I. The yields of T-I and T-II were 9% and 11%, respectively, while they were 24% and 6% for G-I and G-II. T-II showed approximately 30 - 40% lower specific activity than T-I. In several preparations a considerable loss of T-II was observed during electrophoresis. Recrystallization of the isolated fractions did not yield higher specific activities.

Table 1. Purification of G-I.
Starting material: 50 g of coli $BT2^r$ paste.

Steps	Protein	Total activity	Specific activity
	mg	Units	Units/mg Protein
I	1900	990	0.52
II	1280	890	0.7
III	680	816	1.2
IV	360	720	2.0
V	104	468	4.5
VI	19	304	16.0
VII	10	270	27.0
VIII.....	8	242	30.2

One unit of GTPase is the activity liberating 1 μmole of P_i in 10 min at 30°.

Table 2. Purification of T-I.
Starting material: The same as in Table I.

Steps	Protein	Total activity	Specific activity
	mg	Units	Units/mg Protein
I	1900	1390	0.73
II	1280	1280	1.0
III	680	1090	1.6
IV	360	756	2.1
V	104	520	5.0
VI	10.4	185	18.0
VII	4.4	144	33.0
VIII.....	3.2	120	37.5

A unit of polymerization activity was taken as the amount of enzyme which polymerizes 1 mμmole phenylalanine in the presence of a saturating amount of the complementary G factor in 10 min at 30°.

It is not yet clear whether G-II and T-II are merely a result of interactions between G-I and T-I or whether they represent different enzymatic

forms. Both T-I and T-II bind GTP (19) as has recently been observed for similar polymerization factors (20,21,22,23). Attempts to fractionate the T-activity isolated by this procedure into the two complementary fractions (T_u and T_s) identified by Lucas-Lenard and Lipmann (5) have not yet been successful. Physico-chemical properties and the physiological role of the crystallized enzymes are presently under investigation.

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