STUDIES OF AMINO ACID INCORPORATION WITH PURIFIED RIBOSOMES AND SOLUBLE ENZYMES FROM ESCHERICHIA COLI*

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Studies on amino acid incorporation (in vitro) into bacterial ribosomes in cell-free system have been carried out in several laboratories (Schatschabel and Zillig, 1959, Lamborg and Zamecnik, 1960, Tissieres et al., 1960).

In this communication, procedures for purification of ribosomes and soluble 'enzymes' from E. coli will be described. Both ribosomes and enzyme preparations obtained are stable after freeze-drying with respect to their amino acid incorporating activity. A requirement for GTP for maximal amino acid incorporation was also demonstrated with certain fractionated soluble enzymes.

Preparation of ribosomes and enzymes

E. coli strain B(H) was grown in peptone-glucose medium with shaking at 37°C Cells were harvested at late log phase (5-6 x 10^8cells/ml), rapidly chilled with ice, collected and washed by centrifugation. These and subsequent procedures were carried out at 0-5°C. Pellets were suspended in 3 times the volume of 0.03M Tris buffer (pH7.8) containing 7.5 x 10^{-3}M $MgCl_2$ (TBM buffer), and exposed to 10KC sonication for 7min. After two successive centrifugations at 40,000g for 20min., the extract was divided into the small granule fraction (pellet) and the soluble fraction (supernatant) by centrifugation at 105,000g for 150min.

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Soluble enzymes were prepared as follows: After the last centrifugation described above, upper 2/3 (soluble fraction) of the tubes were pipetted off, diluted with equal volume of TBM buffer and adsorbed on TBM-bufferized DEAE cellulose column. The column was washed with TBM buffer to remove unadsorbed amino acids and low molecular materials, and eluted with TBM buffer containing 0.20M NaCl. Most of the S-RNA and DNA remained adsorbed on the column and were separated from soluble enzymes at this stage. The pH of the 0.2M NaCl-eluate was brought to 4.7 with acetic acid, and the resulting precipitate was collected by centrifugation. The precipitate was dissolved in TBM buffer, and the pH of the solution was readjusted to 7.8. This preparation contains about 20% protein and 5-10% RNA of the original soluble fraction, and will be referred to as 'enzyme'. The 'enzyme' preparation exhibited the activity of incorporating amino acids into protein when added to ribosomes. The apparent specific activity of the 'enzyme' increased 4-6 times at this stage as compared to that of the unfractionated soluble fraction.

Ribosomes were prepared from the small granule fraction. The pellet was resuspended in TBM buffer, and subjected to 40,000g centrifugation for 20min. to remove aggregated materials. Sodium deoxycholate was added to the supernatant at the final concentration of 0.7%. The mixture was immediately centrifuged at 105,000g for 120min., and the pellet was resuspended in TBM buffer. The 40,000g and 105,000g centrifugations were repeated, and the final pellet obtained was resuspended again into TBM buffer. Finally, a small amount of aggregated material was removed by 40,000g centrifugation, and the supernatant with slight opalescence was obtained (ribosomes). As will be seen in Fig.1, non-ribosomal materials originally present in the small granule fraction were almost completely removed at this stage.

Aliquots of 'enzymes' and ribosomes were immediately tested for their amino acid incorporating activity, and remainders were freeze-dried at 0°C. The freeze-dried materials were stored at -15°C. After 4 months storage, 80-90% of amino acid incorporating activity was retained as compared to fresh sample.

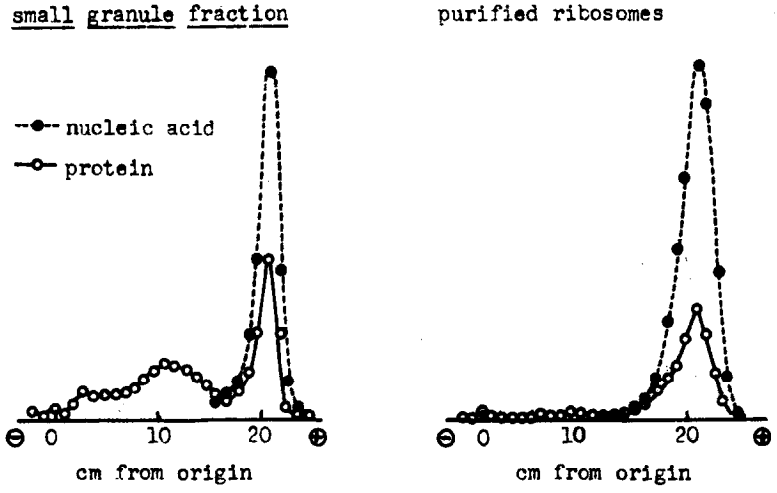

Fig. 1 The starch zone electrophoretic patterns of the small granule fraction and purified 'ribosomes' (see text). Samples were run in 1 x 2.5 x 45cm troughs with starch bufferized with TBM buffer at 11V/cm, 6mA/cm^2 for 15 hr at 5°C. After completion of the run, the starch was cut into 1cm segments, eluted with TBM buffer and proteins (Lowry's method) and nucleic acids (absorption of 260 mu) were determined.

Incorporation of amino acids

The optimal system for incorporating amino acids are presented in the legend of Table 1, and the results are shown in the table. Of the conditions tested, Mg^{++} concentration had a marked influence on the incorporation, the optimum range being 0.005-0.01M. An energy generating system and ATP were also essential, and the optimum concentration of ATP was found to be 0.2-0.5umoles. Saturation for the 'enzyme' was attained at 0.3-0.5mg, when 2mg of ribosomes were used. When concentration of amino acids increased, a roughly linear increase of incorporation was observed, thus 0.7ug of amino acids (0.08umoles each of 17 amino acids) was employed. Incorporation was inhibited with RNase, but not with DNase. Details of the experiments will be published elsewhere.

Requirement for GTP

The requirement for GTP in amino acid incorporation system of E. coli has been somewhat controversial; Lamborg et al. (Lamborg and Zamecnik, 1960) reported its slight stimulating effect, while others (Schatschabel and Zillig, 1959,

TABLE I

Incorporation of amino acids into proteins with cell-free system of E. coli

System	Incorporation activity (% of complete system)
Complete*	100
" minus Ribosomes	10
" Ribosomes replaced by boiled ribosomes	20
" minus Enzymes	15
" Enzymes replaced by boiled enzymes	20
" minus ATP	53
" minus Creatine phosphate, Creatine kinase	19
" plus Mg^{++}(to 2 x 10^{-2}M)	42
" plus EDTA (20umoles)	8
" plus RNase (1ug)	5
" plus DNase (20ug)	96
" Ribosomes and Enzymes replaced by freeze-dried ribosomes and freeze-dried enzymes	85
" Zero time	2

* Complete system (0.75 ml) contained: Tris 22.5umoles pH 7.8, KCl 45umoles, $MgCl_2$ 5.6umoles, ATP 0.5umoles, Creatine phosphate 8umoles, Creatine kinase 0.1mg, 17 amino acids mixture (0.08umoles each) 0.2mg, C^{14}-amino acids mixture from chlorella hydrolyzate (4.5mc/mMC) 3.4 x 10^5cpm), Ribosomes 2mg, Enzymes 0.6mg. Incubation was carried out by standing at 37°C for 20min. After incubation, 1.5mg ovalbumin was added, and the reaction was stopped by addition of 5ml of 3% perchloric acid (PCA). The reaction tubes were heated at 90°C for 30min., and the extraction with hot PCA was repeated. The residue obtained was washed three times with PCA, with 75% alcohol (neutralized with Na-acetate), and then with alcohol-ether mixture at 45°C. The washed residue was solubilized in dilute ammonia, transferred to steel planchets and counted by windowless gas flow counter. The average activity of incorporation of amino acids for complete system was 300cpm (about 0.15ug amino acids incorporated per 2mg ribosomes).

Tissieres et al., 1960) failed to see any effect. In an attempt to clarify this point, 'enzymes' were further fractionated by DEAE cellulose column, using NaCl gradient concentration in TBM buffer. Activity of 'enzyme' was then tested in the proportional region of 'enzyme' concentration with each fraction. As will be seen in Fig.2, 'enzyme' activity could be eluted as a single broad peak, and the stimulating effect of GTP was observed for the slow-eluting half of the peak. Therefore, it is clear that at least a part of the reactions catalyzed by the 'enzyme' requires GTP for its maximal activity, although the precise nature of the GTP requirement is not clear at the present time.

In conclusion, when properly fractionated soluble enzymes were used, incorporation of amino acids into protein of E. coli requires ribosomes, enzymes, ATP,

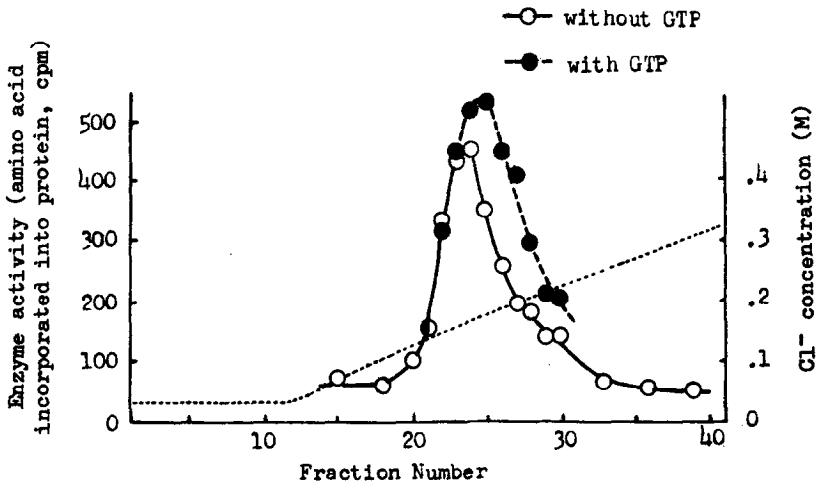

Fig.2 Fractionation of 'Enzymes' by DEAE cellulose column chromatography and the effect of GTP on amino acid incorporation into protein. 55mg portion of 'enzyme' were adsorbed on DEAE cellulose column (1.0 x 6cm, bufferized with TBM buffer), washed with TBM buffer and eluted with concentration gradient from zero to 0.35M of NaCl in TBM buffer. (Elution speed, 5ml/hr, 1.3ml/fraction). All the operations were carried out at 5°C. 'Enzyme' activity was tested with each eluate with or without 0.1umole GTP under the conditions shown in the legend of Table 1, except that non-radioactive amino acid mixture was omitted, and 3 mg freeze-dried ribosomes were used. Recovery of 'enzyme' activity was about 60%. Free GTP was completely eluted between fraction Nos.14 and 19 under these conditions.

an energy generating system, Mg^{++}, and GTP, closely analogous to the system of rat liver ribosomes and soluble enzymes (Keller and Zamecnik, 1956). It seems that the remarkable stability of the E. coli ribosomes and 'enzymes' gives great convenience in further studies concerning the mechanisms of bacterial protein synthesis in general.

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