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INITIATION, ELONGATION AND TERMINATION OF POLYPEPTIDE SYNTHESIS IN CELL-FREE SYSTEMS FROM POLYAMINE-DEFICIENT BACTERIA

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SUMMARY. Protein synthesis in a polyamine-auxotrophic mutant of E.coli cultivated in the absence and presence of putrescine has been studied. The ratio of in vivo polypeptide synthesis under both conditions was the same as that obtained with cell-free extracts derived from polyamine-depleted and supplemented bacteria. The markedly reduced activity in systems from polyamine starved cells is most probably due to a poorly efficient initiation step. The estimation of nascent peptide chains and their release from tRNA support the idea that polypeptide elongation and termination processes do not have decreased rates in systems from polyamine-depleted bacteria.

The involvement of polyamines in different steps of protein synthesis is now well established. Many investigations carried out with cell-free systems from bacteria and eukaryotic cells have indicated that polyamines increase aminoacyl-tRNA formation (1-2), initiation of protein synthesis (3-4), the rate of polypeptide chain elongation (5-7) and the fidelity of translation (8-9).

Recent studies with tRNA crystals (10) and polyamine-deficient mutants of E.coli (11-12) have shown that these organic cations are essential for the normal structure and function of tRNA molecules and ribosomal particles.

We have previously reported that polyamine auxotrophs of E.coli cultivated in the absence of putrescine showed a very low rate of growth and contained defective 30S ribosomal subunits (12). These abnormal particles were responsible for the decreased translation obtained in vitro with poly U and natural messenger RNA (3,12,13) and for the reduced capacity of the AUG-dependent binding of formyl-methionyl-tRNA to ribosomes (3).

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In the present paper we have compared the in vivo protein synthesis in a polyamine-requiring strain of E.coli grown in the absence and presence of putrescine with the translation directed by MS2 phage RNA in cell-free systems obtained from the same mutant. Our aim was to investigate in further detail whether the reduced protein synthesis occurring in polyamine-starved cells can be accounted for a deficiency in initiation, elongation or termination steps of polypeptide synthesis.

MATERIALS AND METHODS

Putrescine dihydrochloride was obtained from Sigma; MS2 phage RNA from Miles and E.coli W stripped tRNA from General Biochemicals. L-[^{14}C]Phenylalanine (527 Ci/mole) was purchased from New England Nuclear Corporation and L-[^{3}H]leucine (133 Ci/mmole) and [^{3}H]puromycin (5.7 ci/mmole) from the Radiochemical Centre, Amersham. Formyl-[^{3}H]methionyl-tRNA (345 cpm/pmole) was kindly provided by Dr.M.Garcia-Patrone.

The polyamine auxotroph E.coli BGA 8 (thi leu thr speB speC rps L^{+}) (9) has been used throughout. The growth medium and conditions for polyamine starvation have been described previously (14). Bacteria were collected after slow cooling of cultures at the exponential phase of growth.

The S_{30} supernatant fractions were obtained as already described (11).

Protein synthesis "in vivo" and "in vitro". The rate of protein synthesis in vivo was followed in aliquots of exponential cultures grown in the absence or presence of putrescine. After addition of radioactive phenylalanine samples were taken at the times indicated in each case and incubated for 20 minutes at 30°C with an equal volume of 1 M NaOH containing unlabeled phenylalanine and albumin (50 μg/ml). Proteins were precipitated with cold trichloroacetic acid (10% final concentration), filtered through Whatman GF/C filters, washed and counted in a scintillation spectrometer.

Polypeptide synthesis in cell-free systems programmed with MS2 phage RNA was measured as described previously (3).

MS2 phage RNA-induced binding of formyl-methionyl-tRNA to ribosomes. The incubation mixtures were similar to those used for polypeptide synthesis with the addition of 56 pmoles of formyl-[^{3}H]methionyl-tRNA instead of amino acid mixture. After 20 min incubation at 34°C, 3 ml of cold 0.1 M Tris-HCl buffer, pH 7.4 containing 20 mM magnesium acetate and 50 mM KCl were added and the resulting suspensions filtered through Millipore membranes (HAWP, 0.45 μm pore size). After washing three times with 3 ml of the same buffer, Millipore filters were dried and counted.

Peptidyl-[^{3}H]-puromycin formation. The standard incubation mixture for polypeptide synthesis induced by MS2 phage RNA and containing unlabeled amino acids was used. After different periods of incubation, [^{3}H]puromycin was added (12 μM) and the reaction was continued for 5 minutes more. Peptidyl-[^{3}H]-puromycin was precipitated and measured as indicated by Pestka (15).

Assay for tRNA-bound polypeptides. Nascent peptide chains bound to tRNA were measured by precipitation with cetyltrimethylammonium bromide as described by Hobden and Cundliffe (16).

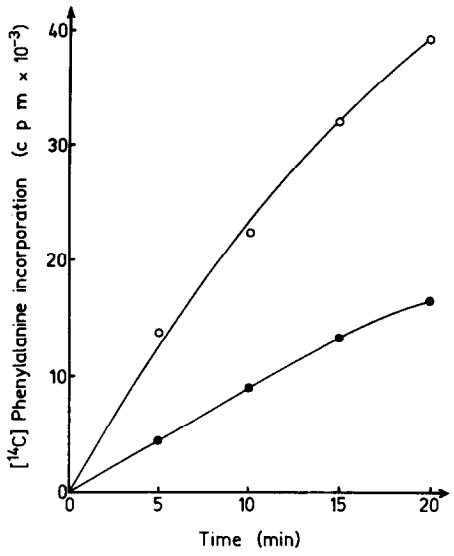

Figure 1. Kinetics of protein synthesis in vivo. Cultures of E.coli BGA8 grown in the presence and absence of putrescine were labeled with [^{14}C]phenylalanine (0.06 μCi/ml). After the indicated times 1 ml aliquots were taken and treated as detailed in Materials and Methods to measure the radioactive polypeptides formed. Symbols: ● and ○ correspond to bacteria cultivated in the absence and presence of putrescine, respectively.

RESULTS AND DISCUSSION

Cultures of E.coli BGA 8 growing exponentially in the presence and absence of putrescine were labeled with radioactive phenylalanine and total protein synthesis was measured during a 20 minutes period. Fig. 1 shows that the rate of protein synthesis in polyamine supplemented bacteria was about two-fold higher than in putrescine-starved cells.

In order to compare the in vivo protein synthesis with the in vitro translation we have obtained S_{30} extracts from both polyamine unstarved and depleted bacteria and these preparations were used to follow the time course of polypeptide synthesis directed by MS2 phage RNA. We were able to show that the protein synthesizing activity of cell-free systems from polyamine-supplemented bacteria was again about two-fold higher than in extracts from putrescine depleted cells (Fig. 2). These results, similar to those reported previously (3,14), indicate that the ratio between in vitro translation activities in both systems is almost identical to the relative value obtained in experiments performed in vivo.

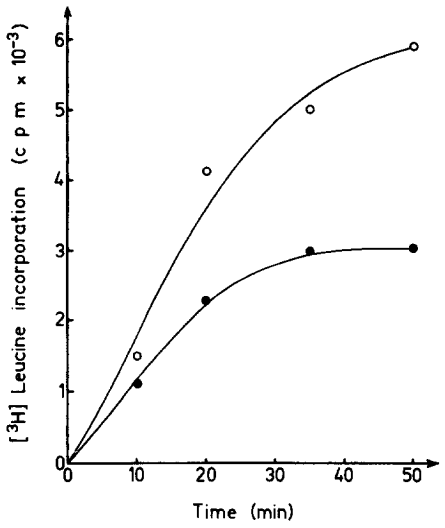

Figure 2. Time course of leucine incorporation into polypeptides directed by MS2 phage RNA in S_{30} extracts of putrescine-depleted and supplemented bacteria. Incubation mixtures as described in Materials and Methods with 0.6 A_{260} units of S_{30} extracts and [^{3}H]leucine (8 μM, 200 μCi/ml). Symbols as in Fig.1.

Since the initiation, elongation and termination steps of the translation process can be studied separately in experiments carried out in vitro, we have investigated each of these reactions with the purpose of finding out whether all of them are altered in extracts from polyamine-starved cells or only one is responsible for the decreased protein synthesis.

Initiation was measured by the binding of formyl-methionyl-tRNA to ribosomes induced by the natural messenger MS2 phage RNA. The results given in Table I indicate that the initiation complex formation is two-fold higher in S_{30} extracts from putrescine-supplemented bacteria.

The active ribosomes in protein synthesis are those which bear nascent polypeptide chains. In order to estimate the amount of active ribosomes engaged in translation we have measured the growing peptide chains still attached to tRNA by using the reaction with puromycin. This antibiotic releases more than 90% of the nascent protein chains after 5 minutes incubation under the conditions used for in vitro translation. Since only one molecule of puromycin is added per peptide chain, it is possible to quantitate the amount of nascent

TABLE I

MS2 phage RNA-Dependent Binding of Formyl-[^{3}H]Methionyl-tRNA to Ribosomes from Polyamine-Starved or Unstarved Bacteria.

Ribosomes source	f[^{3}H]met-tRNA bound (pmoles/mg ribosomes)
Polyamine-depleted bacteria	7.9
Polyamine-supplemented bacteria	16.3

Standard reaction mixtures for polypeptide synthesis were used with 0.6 A_{260} units of S_{30} extracts. Formyl-[^{3}H]met-tRNA (56 pmoles; 19.300 cpm) was added instead of amino acids. The blank values obtained in the absence of messenger RNA were subtracted in each case.

polypeptides by using unlabeled amino acids and adding radioactive puromycin at different times. Fig. 3 shows the time course of leucine incorporation into radioactive peptides directed by MS2 phage RNA (Fig. 3 A) and the corresponding amounts of peptide chains released by puromycin during the reaction (Fig. 3 B). From these results we have calculated that the percentages of active ribosomes involved in translation in cell-free extracts derived from

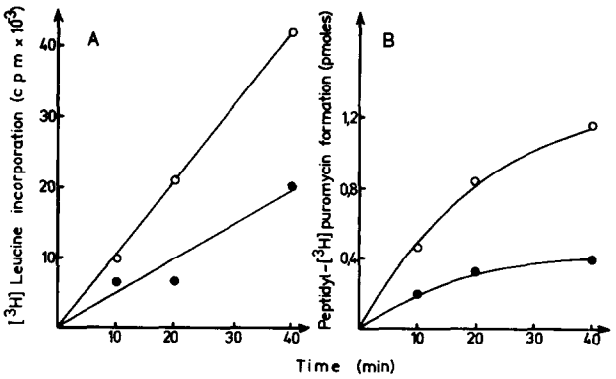

Figure 3. Kinetics of polypeptide synthesis and peptidyl-puromycin formation in cell-free systems from polyamine-starved and unstarved cells programmed by MS2 phage RNA. A) Leucine incorporation into polypeptides carried out as in Fig. 2. B) Peptidyl-puromycin formation after addition of [^{3}H]puromycin (12 μM 66 μCi/ml) to standard reaction mixtures for polypeptide synthesis previously incubated with unlabeled amino acids for the indicated times. All other details as in Materials and Methods. Symbols as in Fig. 1.

TABLE II

Percentage of Active Ribosomes in Cell-free Systems from Polyamine-Depleted and Supplemented Bacteria Programmed by MS2 phage RNA.

S_{30} source	Active Ribosomes %	[^{3}H]leucine incorporation (cpm x 10^{-3})
Polyamine-starved cells	7.6	6.2
Polyamine-unstarved cells	19.3	20.4

Peptidyl-puromycin formation and total leucine incorporation were measured as indicated in Materials and Methods and in Fig. 3. The amount of active ribosomes, which are equivalent to the corresponding number of nascent peptide chains released by puromycin, were expressed as percentages of the total amount of ribosomes used in the assay.

polyamine-starved and unstarved bacteria were proportional to the corresponding total amounts of amino acid incorporation into polypeptides (Table II). This fact strongly suggests that the elongation rate in both systems is approximately the same; if the rate of peptide chain elongation were higher or peptides were longer in systems from polyamine-supplemented cells we should have found a much higher total amino acid incorporation in this system.

Termination of protein synthesis can be estimated by the natural release of peptide chains during translation. Cetyltrimethylammonium bromide precipitates polypeptides bound to tRNA but not free polypeptides (17). Therefore we have been able to calculate the amount of polypeptides released from ribosomes by subtracting radioactivity corresponding to cetyltrimethylammonium bromide-insoluble peptides from total radioactivity precipitated by trichloroacetic acid. Fig. 4 shows that the percentages of peptides released at different times during the translation of MS2 phage RNA were approximately the same in both cell-free systems obtained either from putrescine-starved or unstarved bacteria.

The results described in this communication confirm previous conclusions that the reduced polypeptide synthesis observed in extracts from polyamine-

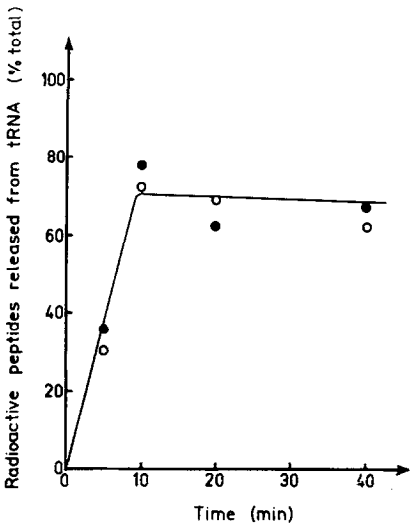

Figure 4. Time course of polypeptide chains release in cell-free systems from putrescine-depleted and supplemented bacteria programmed by MS2 phage RNA. Assays were performed as described in Materials and Methods. Aliquots were taken by duplicate at the indicated times and total radioactivity in trichloroacetic acid and cetyltrimethylammonium bromide-insoluble materials were measured. Released peptides were calculated as described in the text and expressed as percentages of the corresponding total radioactivity precipitated by trichloroacetic acid. Symbols as in Fig. 1.

depleted bacteria is mainly due to a low efficiency of the initiation step. The elongation rate and termination of peptide chains appear to be normal in this system, at least for the translation of a low molecular weight natural messenger as that of MS2 phage coat protein. This conclusion does not exclude that when longer messengers are involved as it also occurs in vivo, a decreased elongation rate could be observed probably due to the premature release of some unfinished polypeptides caused by a reduced fidelity of translation (9,18).

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