PROTEIN SYNTHESIS AND RIBOSOMAL DISTRIBUTION IN A POLYAMINE AUXOTROPH OF ESCHERICHIA COLI: STUDIES IN CELL-FREE SYSTEMS

G. Echandi and I. D. Algranati

Instituto de Investigaciones Bioquímicas "Fundación Campomar" and Facultad de Ciencias Exactas y Naturales, Obligado 2490, Buenos Aires (28), Argentina

Received November 13,1974

SUMMARY: Phenylalanine incorporation into polypeptides induced by poly (U) is markedly reduced in extracts obtained from polyamine depleted bacteria, and this effect, totally independent of RNA synthesis, is related to some deficiency at the level of the ribosomal particles and/or the factors bound to them. The ribosomal profiles corresponding to polyamine starved cells change when cultures are supplemented with putrescine.

The polyamines are organic cations which are present practically in all living cells. Although their exact physiological roles are still not well understood, many authors have indicated that these substances participate in a wide variety of processes, such as stabilization of membranes and subcellular structures (1, 2), osmotic adaptation (3) and biosynthesis of nucleic acids and proteins (4, 5).

Recent studies carried out with Escherichia coli mutants blocked in the biosynthesis of putrescine and spermidine have suggested that at least some of the polyamines effects on translation are not dependent on RNA synthesis (6, and Algranati, I. D., Echandi, G. and Maas, W. K., manuscript in preparation). This conclusion is further confirmed by the experiments reported in this paper, which describes the results obtained studying polypeptide synthesis and ribosomal distribution in cell-free systems derived from bacteria grown in the absence and presence of polyamines. Furthermore the altered site of the translation machinery in starved cells was investigated.

MATERIALS AND METHODS

Putrescine dihydrochloride and sucrose (ribonuclease-free) were purchased from Schwarz-Mann (Orangeburg, N.Y.); poly (U) as K^+ salt, was obtained from Sigma; E. coli stripped tRNA from General Biochemicals (Chagrin Falls, Ohio), and [^{14}C]phenylalanine from New England Nuclear Corp.

Bacterial strain and culture conditions. Escherichia coli MA 261 Thr^-, Leu^-, Ser^-, Thi^-, kindly supplied by Dr. W. K. Maas, has been used in all the experiments. This strain is in addition a double mutant deficient in

the enzymes agmatine ureohydrolase (AUH^-) and the constitutive ornithine decarboxylase (OD_c^-). Therefore E. coli MA 261 can neither synthesize putrescine nor spermidine and requires an exogenous polyamine supply for growth (W. K. Maas, personal communication).

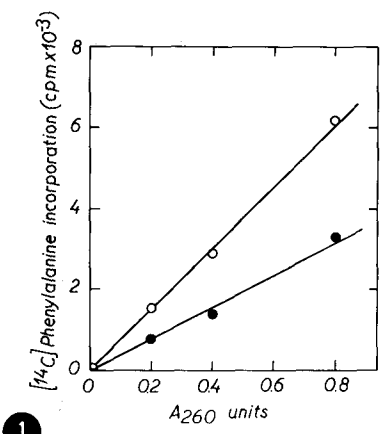
The growth media and the procedure for polyamine starvation of bacteria will be described elsewhere. After the starvation E. coli cells were grown in the absence or presence of putrescine (MMO or MMOP media, respectively)[1].

Preparation of cell-free systems. Exponentially growing cultures were harvested after slow cooling and cell-free extracts were obtained by gentle lysis as previously described (7), or by grinding in a mortar for 5 min with 2 g of alumina per g of wet cells. After suspension of the mixture in Buffer I containing 10 mM Tris-HCl, pH 7.8, 5 mM magnesium acetate, 60 mM NH_4Cl and 6 mM 2-mercaptoethanol (3-4 ml per g of wet bacteria), alumina and cell debris were removed by centrifugation for 15 min at 20,000 x g. The resulting supernatant fluid was incubated with 3 μg DNAse per ml at 0°C during 5 min and after a second centrifugation for 30 min at 30,000 x g the supernatant liquid (S_{30}) was collected and dialyzed during 5 h against 500 volumes of Buffer I. In some cases S_{30} preparations were centrifuged for 160 min at 150,000 x g and the upper two thirds of the supernatant fluid (S_{150}), were collected. The pellet of ribosomes was resuspended in a small volume of Buffer I. The supernatant fractions S_{30} and S_{150}, as well as the ribosomal suspension retained their activity for at least several weeks on storage at -70°C.

Ribosomal distribution analysis. The extracts obtained by enzymatic lysis or by grinding of bacteria were layered on top of 15-40% (w/v) linear sucrose density gradients containing 20 mM Tris-HCl buffer, pH 7.8, 10 mM magnesium acetate and 50 mM KCl. After centrifugation for 90 min at 45,000 rpm in a Spinco SW 65 rotor, gradients were analyzed by monitoring at 254 nm with an ISCO ultraviolet analyzer.

In vitro synthesis of polypeptides and phenylalanine activation. The standard reaction mixture for polyphenylalanine synthesis contained in a total volume of 0.1 ml: Tris-HCl, pH 7.8, 50 mM; ATP, 1 mM; GTP, 0.02 mM; phosphoenolpyruvate, 5 mM; pyruvate kinase, 3 μg; NH_4Cl, 60 mM; stripped E. coli tRNA, 40 μg; [^{14}C]phenylalanine (spec. act. 50 Ci/mole), 0.01 mM; poly (U), 50 μg, and magnesium acetate and S_{30} extracts as indicated in each case. S_{150} supernatant fluid and ribosomal suspension were used in some experiments

[1] Abbreviations: MMO, minimal medium without putrescine; MMOP, minimal medium with putrescine.



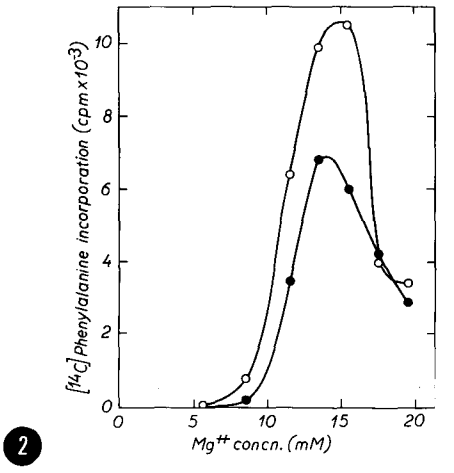


Fig. 1. Phenylalanine incorporation induced by poly (U) in cell-free extracts of E. coli MA 261 cultivated under different conditions. Reaction mixtures were as described in Materials and Methods with 14 mM magnesium acetate and the amounts indicated of S_{30} extracts (expressed in A_{260} units). The incubation time was 30 min. Symbols: ● and ○ correspond to extracts from bacteria grown in MMO and MMOP media, respectively.

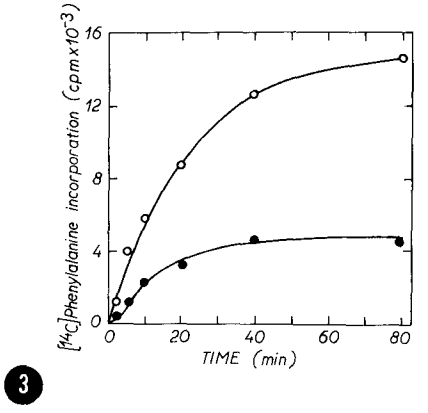
Fig. 2. Effect of Mg^{++} concentration on polyphenylalanine synthesis in extracts from starved or unstarved bacteria. Standard reaction mixtures were used with 0.4 A_{260} units of S_{30} extracts and the indicated Mg^{++} levels. All other details and symbols as in Fig. 1.

instead of S_{30} extracts. The complete mixture without poly (U) was preincubated for 3 min at 37°C and after the addition of the mRNA the reaction was carried out at the same temperature during the indicated times. The reaction was stopped by adding cold trichloroacetic acid (final concentration, 6%). After heating at 90°C for 15 min the precipitate was collected on Millipore filters, washed with cold 5% trichloroacetic acid, dried and counted in a Packard scintillation counter.

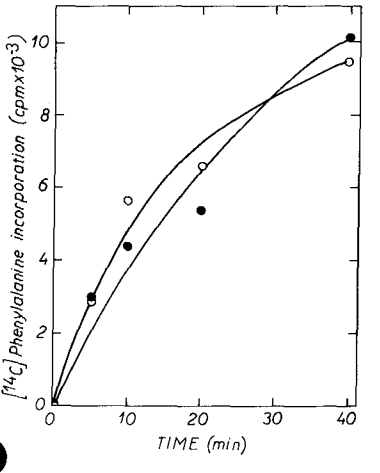
In order to determine the Phe-tRNA formation the standard reaction mixture, without poly (U), was used, and the radioactivity of cold 5% trichloroacetic acid insoluble material was measured.

RESULTS AND DISCUSSION

In vivo studies carried out recently in our laboratory with E. coli MA 261 have indicated that the amino acid incorporation into proteins increased when bacteria grown under conditions of polyamine starvation were supplemented with putrescine. The enhancement of protein synthesis due to the addition of poly-



3



4

Fig. 3. Kinetics of Phe incorporation by S_{30} extracts from starved or unstarved cells.
Reaction mixtures as described in Materials and Methods with 14 mM magnesium acetate and 0.4 A_{260} units of each bacterial extract.
Symbols as in Fig. 1.

Fig. 4. Kinetics of Phe activation in S_{30} extracts from starved or unstarved cells.
Reaction mixtures as detailed in Materials and Methods and Fig. 3.
Symbols as in Fig. 1.

---

amine was observed even in the presence of rifampicin which is able to inhibit RNA formation almost completely (Algranati, I. D., Echandi, G. and Maas, W.K., manuscript in preparation). These results strongly suggest that the polyamines stimulatory effect on protein synthesis is independent of transcription. The use of an *in vitro* system with synthetic mRNA enabled us to dissect the processes of transcription and translation. Experiments carried out in this way permitted us to confirm our hypothesis. The polypeptide synthesis in cell-free systems prepared from bacteria grown in the presence of putrescine was 2 to 5 fold higher than the corresponding activity of extracts derived from polyamine-depleted cells. Fig. 1 shows the phenylalanine incorporation induced by poly (U) with different amounts of S_{30} extracts obtained from polyamine starved or unstarved bacteria. This effect could be due to differences in Mg^{++} requirements for maximal activity. However, this was not the case, since the Mg^{++} level giving maximal incorporation was 13 to 15 mM in both extracts (Fig. 2). It is relevant to mention that several authors (8, 9) have found a decrease of the optimal Mg^{++} concentration required for polypeptide synthesis when spermidine was added to the *in vitro* systems.

Table I. Poly (U)-dependent phenylalanine incorporation catalyzed by reconstituted systems containing ribosomes and S_{150} supernatant fractions from starved or unstarved bacteria

The standard reaction mixtures contained ribosomal suspension (0.2 A_{260} units) and S_{150} supernatant fluid (15 μg of protein) prepared from cells cultivated in MMO or MMOP as indicated in each experiment. Mg^{++} concentration was 14 mM and all other components were as described in Materials and Methods. The incubation time was 30 min. The blank values obtained in the absence of poly (U) were subtracted in each case.

	Ribosomes		S_{150}		Polypeptide synthesis (pmoles of Phe incorporation)
	from bacteria grown in medium				
	MMO	MMOP	MMO	MMOP	
Expt. 1	+	-	+	-	48.4
	+	-	-	+	59.6
	-	+	+	-	143.5
	-	+	-	+	136.0
Expt. 2	+	-	+	-	25.0
	+	-	-	+	46.4
	-	+	+	-	70.5
	-	+	-	+	97.8

The time course of phenylalanine incorporation indicated that protein synthesis had a lower initial velocity and levelled off earlier in the extracts of cells cultivated in MMO medium (Fig. 3). These effects cannot be attributed to differences in the amino acid activation step, since this process had almost identical kinetics in both systems, as can be seen in Fig. 4.

The effect of bacterial polyamine starvation on polypeptide synthesis in cell-free systems could be related to some deficiency at the level of ribosomal particles, the soluble factors present in the supernatant fraction or both. In order to elucidate this problem the S_{30} extracts were centrifuged and ribosomes were separated from the supernatant fluids. Afterwards the polyphenylalanine synthesis was measured in standard reaction mixtures combining ribosomes and S_{150} fractions derived from starved and unstarved cells. The values given in Table I show that the stimulation of polypeptide synthesis observed in some experiments depends exclusively on ribosomes, whereas in other preparations this effect depends on ribosomal particles as well as on S_{150} supernatant fractions. These results could be explained if we assume that the polyamine starvation is able to induce an alteration of a ribosomal component or factor that in some preparations might be partially released to the supernatant fluid.

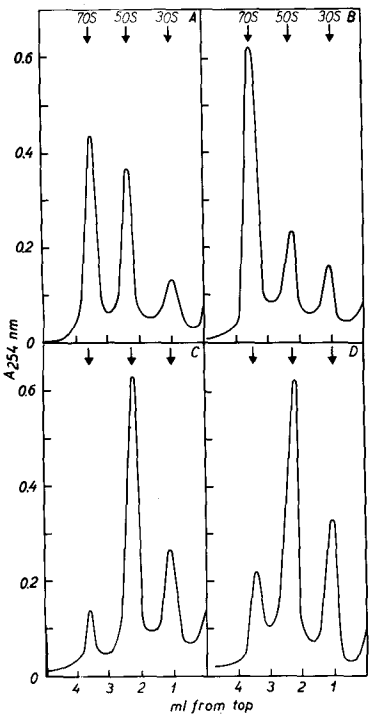


Fig. 5. Effect of polyamine addition to the cultures on the ribosomal profiles of bacterial lysates and S_{30} fractions.
Cell extracts (about 0.6 A_{260} units) were analyzed after sucrose gradient centrifugation. A and B are the ribosomal patterns corresponding to lysates, and C and D to S_{30} extracts. A and C are the profiles of samples obtained from starved bacteria; B and D correspond to cells grown in the presence of putrescine.

From Table I it can also be concluded that ribosomes obtained either from polyamine starved or unstarved bacteria are functionally different. This fact led us to study the distribution of ribosomal particles contained in lysates and S_{30} extracts prepared from cells grown in both conditions. The profiles corresponding to enzymatic lysates represent the intracellular ribosomal distribution, while those corresponding to the S_{30} extracts obtained by grinding of bacteria, give a good picture of ribosomal particles present in the cell-free systems used in our polypeptide synthesis assays. The sucrose gradient analysis shown in Fig. 5 indicated that lysates obtained from polyamine depleted bacteria contained relatively low amounts of 70S ribosomes and 30S particles in comparison to the high 50S peak. On the other hand lysates from cells cultivated in MMOP medium showed a marked increase of 70S monomers and a normalization of the relative amounts of both subunits. Similar patterns with respect to the relationship between subparticles were observed in S_{30} extracts (Fig. 5C and 5D); however, in these preparations most

of 70S ribosomes disappeared due to their dissociation during the cell rupture procedure. The lower total ribosomal content observed in the profiles corresponding to starved bacteria is compensated by a higher absorbancy in the supernatant (not shown in figures).

Our results demonstrate that *in vitro* protein synthesis is markedly lower in cell-free extracts prepared from polyamine depleted bacteria. The initiation step as well as the elongation of polypeptides seem to be altered. Furthermore the ribosomal distribution pattern is abnormal, suggesting that the biosynthesis and/or the assembly of 30S subunits are somehow damaged during polyamine starvation. This possibility is at present under investigation in our laboratory.

Although the effect of intracellular polyamine levels on protein synthesis seems to be related to the ribosomal fraction (Table I), more experiments are required to conclude that the observed alteration in polypeptide synthesis is a direct consequence of some change occurring in ribosomes. On the other hand the present studies on *in vitro* systems clearly indicate that the described effect on translation caused by polyamine starvation of bacteria is independent of the RNA synthesis process.

Acknowledgements. We are grateful to Dr. Luis F. Leloir and all members of the Instituto de Investigaciones Bioquímicas for helpful discussions. This work was supported in part by a grant from the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina). I.D.A. is a career investigator of the latter institution and G.E. is a fellow of the Organization of American States.

REFERENCES

1. Cohen, S. S. (1971) Introduction to the Polyamines. Prentice-Hall, Inc., Englewood Cliffs, N.J.
2. Cohen, S. S., and Lichtenstein, J. (1960) J. Biol. Chem. 235, 2112-2116.
3. Munro, G. F., and Bell, C. A. (1973) J. Bacteriol. 115, 469-475.
4. Stevens, L. (1970) Biol. Rev. 45, 1-27.
5. Bachrach, U. (1973) Function of Naturally Occurring Polyamines. Academic Press, New York.
6. Young, D. V., and Srinivasan, P. R. (1972) J. Bacteriol. 112, 30-39.
7. Algranati, I. D., González, N. S., and Bade, E. (1969) Proc. Nat. Acad. Sci. U. S. A. 62, 574-580.
8. Algranati, I. D., and Lengyel, P. (1966) J. Biol. Chem. 241, 1778-1783.
9. Takeda, Y. (1969) J. Biochem. (Tokyo) 66, 345-349.