Vol. 96, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1980 Pages 54-60

INHIBITION OF POLYPEPTIDE SYNTHESIS INITIATION BY A CHANGE OF Mg^{++} CONCENTRATION IN WHEAT GERM CELL-FREE SYSTEMS

I. D. Algranati

Instituto de Investigaciones Bioquímicas "Fundación Campomar" and
Facultad de Ciencias Exactas y Naturales, Obligado 2490,
1428 Buenos Aires, Argentina

Received June 29,1980

SUMMARY - Polypeptide synthesis programmed by poly(U) and globin mRNA has been studied in cell-free extracts from wheat germ. A two-step reaction with a preincubation at high Mg^{++} levels followed by a second step carried out after a shift to a low Mg^{++} concentration and the addition of labeled amino acids is described. Under these conditions the initiation of polyphenylalanine synthesis can be blocked without affecting the elongation of polypeptide chains. This procedure allows the selective inhibition of polypeptide synthesis initiation without using any drug or antibiotic.

Synthetic and natural messenger ribonucleic acids can be translated in cell-free systems obtained from both bacteria and eukaryotic cells. However, the polypeptide synthesis directed by an artificial messenger as poly(U) occurs at higher Mg^{++} levels due to the requirements for the binding of these messengers to ribosomal particles. This initiation step with synthetic polynucleotides takes place either without the involvement of initiation factors (1, 2) or with the participation of only two of them (3).

The efficient translation of artificial and natural messengers at different optimal Mg^{++} concentrations seems to indicate that the elongation of polypeptide chains has a less stringent requirement for Mg^{++} levels than the initiation process. Therefore, the polypeptide synthesis reaction could be carried out in two separate steps: a) the binding of messenger to ribosomes at a relatively high Mg^{++} level, and b) the subsequent synthesis of polypeptide chains after a shift to a lower Mg^{++} concentration.

0006-291X/80/170054-07$01.00/0

In the present paper we study the poly(U) directed polyphenylalanine synthesis in cell-free systems from wheat germ. We demonstrate that a decrease of Mg^{++} level after the attachment of poly(U) to ribosomes inhibits further initiation of polypeptide synthesis, and that under these conditions only the elongation of polyphenylalanine takes place.

MATERIALS AND METHODS

Raw wheat germ was obtained from Niblack Foods, Inc. (Rochester, N. Y.). Rabbit globin mRNA was kindly provided by Dr. E. Nack. Poly(U) (K salt), wheat germ tRNA and ammonium salt of aurintricarboxylic acid (ATA) were purchased from Sigma. [^{14}C]Phenylalanine (527 mCi/mmol) and [^{35}S]methionine (564 Ci/mmol) were from New England Nuclear Corp.

Wheat germ cell-free extract (S_{23}) was prepared according to a modification of the method described by Marcus et al. (4, 5). Three grams of wheat germ were ground in a precooled mortar with 18 ml of a solution containing 90 mM KCl, 2 mM $CaCl_2$, 1 mM magnesium acetate and 6 mM $KHCO_3$. The resulting suspension was centrifuged for 10 min at 23,000 x g and the supernatant fluid was carefully collected in order to avoid the upper lipid layer. After adding Tris-HCl, pH 7.5, and magnesium acetate to 20 mM and 2 mM final concentrations, respectively, the extract was centrifuged again for 10 min at 23,000 x g. The supernatant liquid was applied to a Sephadex G-25 column (50 x 2 cm) equilibrated with a solution containing 3 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM magnesium acetate and 5 mM 2-mercaptoethanol. After elution with the same buffer the fractions with maximal A_{260} absorbance (about 10-15 ml) were collected, pooled and centrifuged at 23,000 x g for 10 min. The supernatant fluid was fractionated in small aliquots and kept frozen at -70ºC. All operations were performed at 0-4ºC.

In vitro polypeptide synthesis induced by poly(U) or globin mRNA - The standard reaction mixture was carried out in a total volume of 0.05 ml which contained: Hepes-KOH, pH 7.6, 20 mM; ATP, 1 mM; GTP, 0.02 mM; creatine phosphate, 8 mM; creatine phosphokinase, 2 µg; dithiothreitol, 2 mM; 19 unlabeled amino acids excluding phenylalanine or methionine, 0.03 mM each; wheat germ tRNA, 10 µg; S_{23} wheat germ extract, 0.25 to 0.5 A_{260} unit; poly(U), 40 µg, or globin mRNA, 1 µg, were used as messengers, and [^{14}C]phenylalanine, 0.05 µCi, or [^{35}S]methionine, 5 µCi as labeled amino acids, respectively. Potassium acetate and magnesium acetate were added as indicated in each case. The incubation was performed at 27ºC and the reaction was stopped by addition of 1 ml cold water. After 15 min at 37ºC with 0.3 N NaOH containing 2 mg/ml of unlabeled phenylalanine or methionine and 50 µg of bovine serum albumin as carrier, cold trichloroacetic acid was added to 10% final concentration. The precipitate was collected on glass fiber (GF/c) filters, washed with cold 5% trichloroacetic acid, dried and counted in a Packard scintillation counter.

In some experiments complete mixtures without radioactive amino acids were preincubated for 15 min at 27ºC; afterwards, ATA and the corresponding labeled amino acid were added and polypeptide synthesis was measured at different times as described above.

Polyphenylalanine synthesis carried out in two steps - Poly(U) binding to wheat germ ribosomes was performed in 0.025 ml of complete reaction mixtures as described above, but omitting phenylalanine. After preincubation for 15 min at 27ºC (first step), Mg^{++} was adjusted to the desired final levels while all other components (except poly(U) and S_{23} extract which were not added again) were kept at the same initial concentrations. Then, labeled phenylalanine was added and the incubation was resumed at the same temperature in a total volume of 0.075 ml (second step). Aliquots from the reaction mixture were taken at the indicated times to measure the radioactive trichloroacetic acid insoluble material.

RESULTS AND DISCUSSION

Cell-free extracts from wheat germ are able to translate synthetic polynucleotides and natural messengers with good efficiency. The in vitro polypeptide synthesis directed by poly(U) and globin mRNA in wheat germ systems has shown quite different Mg^{++} concentration curves (Fig. 1). The amino acid incorporation directed by globin mRNA was maximal within a narrow range (2 to 3 mM Mg^{++}) whereas polyphenylalanine synthesis showed a broader peak of activity between 4

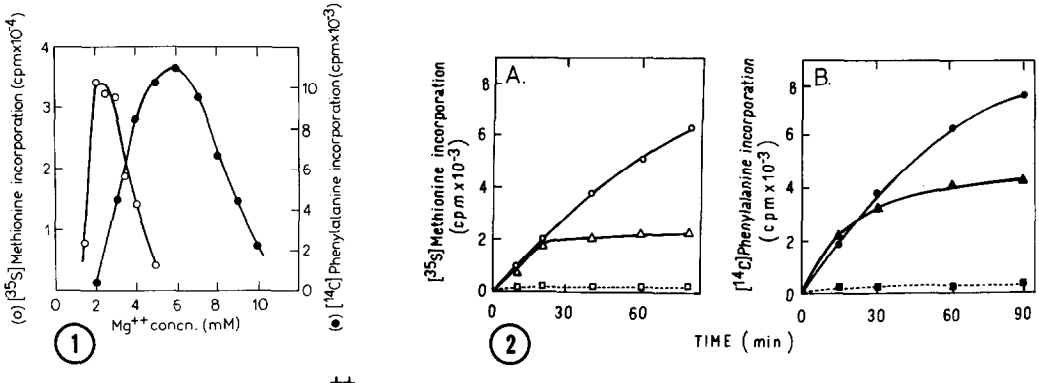
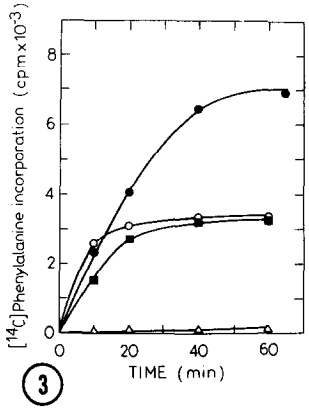


Fig. 1 - Effect of Mg^{++} concentration on polypeptide synthesis induced by poly(U) and globin mRNA in cell-free extracts from wheat germ. Standard reaction mixtures as described in Materials and Methods were used with 0.25 A_{260} unit of S_{23} extracts and the indicated Mg^{++} levels. K^{+} concentrations were 110 mM and 80 mM with poly(U) and natural messenger, respectively. The incubation time was 40 min. Symbols: ● and O correspond to poly(U) and globin mRNA, respectively.

Fig. 2 - Effect of ATA on polypeptide synthesis directed by globin mRNA or poly(U). Reaction mixtures were as described in Materials and Methods and in Fig. 1. A and B show the results of experiments carried out with globin mRNA and poly(U), respectively, at the corresponding optimal conditions for Mg^{++} and K^{+}. The reactions were performed in a single step without any addition (O and ●) or with 6×10^{-5} M ATA added at the beginning of the incubation (□ and ■), and in two steps adding ATA (6×10^{-5} M) and radioactive amino acid at the beginning of the second step (Δ and ▲).

and 7 mM Mg^{++}. These results are probably due to different requirements for the initiation process with natural and synthetic messengers, since the reactions involved in the elongation of polypeptide chains are essentially the same with both kinds of messengers. In fact, it is well known that the participation of initiation factors in the binding of natural messengers to ribosomes gives rise to a concomitant sharp decrease of the Mg^{++} requirement (6, 7). On the other hand the results depicted in Fig. 1 seem to indicate that the elongation of polypeptide chains takes place both at high (6 mM) and low (2 mM) Mg^{++} levels. If this assumption is correct it would be possible to study the elongation of polyphenylalanine independently of the initiation process by a shift of Mg^{++} concentration in the reaction mixtures. It should be mentioned that the amino acid activation under conditions of protein synthesis almost does not change in a wide range of Mg^{++} levels (data not shown).

In order to follow the elongation of polypeptide chains separately from the initiation complex formation we have used ATA, which is a well known inhibitor of the binding of natural and synthetic messengers to ribosomes (8-10). Fig. 2 (A and B) shows that when ATA was added to the reaction mixtures before the messenger, polypeptide synthesis with both poly(U) and globin mRNA was completely inhibited. On the other hand when ATA was added after a 15 min preincubation of the complete mixtures which did not contain labeled amino acids, the inhibitor blocked all subsequent initiations either with poly(U) or globin mRNA. However, ATA did not impair the elongation of polypeptide chains corresponding to the initiation complexes already formed during the preincubation. Once these chains were completed the amino acid incorporation curves levelled off. This kind of kinetics is typical of the inhibition of polypeptide synthesis initiation (11).



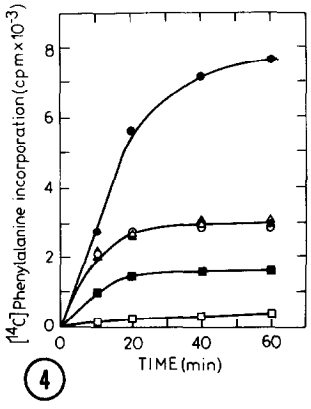


Fig. 3 - Kinetics of polyphenylalanine synthesis after a shift of Mg^{++} concentration or ATA addition. Phenylalanine incorporation assays were carried out a) in a single step reaction at 6 mM (●) or 2 mM Mg^{++} (Δ); b) in a two-step reaction with the following modifications at the beginning of the second step: the addition of 6×10^{-5} M ATA (■), or the shift of Mg^{++} levels from 6 to 2 mM (○). All other conditions as in Materials and Methods and in Fig. 1.

Fig. 4 - Effect of different conditions during the first step of the reaction on the kinetics of polyphenylalanine synthesis. Reactions were performed in two steps with a shift from 6 mM to 2 mM Mg^{++} concentration after the first step carried out at 27ºC for 5 (○), 10 (▲) or 15 min (Δ); at 0ºC for 15 min (■) and at 27ºC for 15 min in the absence of tRNA (□). A control corresponding to the time course of phenylalanine incorporation carried out in a single step at 6 mM Mg^{++} is also shown (●). All other details as in Materials and Methods and in Fig. 3.

The higher level of amino acid incorporation obtained in the absence of ATA is probably due to reinitiation of polypeptide chains with globin mRNA or to a late binding of poly(U) to ribosomes.

Fig. 3 shows the time course of polyphenylalanine synthesis occurring at high (6 mM) or low (2 mM) Mg^{++} concentration, as well as when the reaction was performed in two steps: preincubation at 6 mM Mg^{++} during which a poly(U)-ribosome complex was formed, followed by a second incubation after adding labeled amino acids with a simultaneous decrease of final Mg^{++} levels to 2 mM. At this low Mg^{++} concentration no new initiation complexes can be formed since the one step reaction at 2 mM Mg^{++} showed very little or no polyphenylalanine synthesis (Fig. 3). Therefore under these conditions only the elongation of polypeptide chains took

place during the second step of the reaction. This interpretation is also supported by the almost identical time curve obtained when the reaction was performed in two steps with ATA added at the end of the first step (Fig. 3). The poly(U)-ribosome complex formed during the preincubation, although somewhat unstable at low Mg^{++} levels, could be detected when radioactive synthetic polynucleotide was used and the reaction mixtures were submitted to sucrose gradient centrifugation analysis (data not shown).

A detailed study of the two-step reaction under different conditions has indicated that uncharged tRNA must be present during the first step and that the initiation complex formation occurs better at 27ºC than at 0ºC (Fig. 4). The tRNA requirement for the efficient binding of messengers to ribosomes has been reported previously in bacterial and eukaryotic systems (1, 12).

The results described in this paper with wheat germ cell-free systems demonstrate that a two-step reaction in which the Mg^{++} concentration is decreased after the attachment of poly(U) to ribosomes, is a simple way to inhibit polypeptide synthesis initiation. Although the shift of Mg^{++} concentration has been reported by Shafritz et al. to distinguish the initiation of poly(U)-directed polyphenylalanine synthesis either in the absence or in the presence of initiation factors (3), this procedure has not been used so far to block the initiation step and to measure the elongation of polypeptide chains independently of other reactions involved in the translation process.

Acknowledgements - I am grateful to Drs. L. F. Leloir, N. S. González, S. H. Goldemberg, M. Dankert, M. García-Patrone and all members of the Instituto de Investigaciones Bioquímicas for many helpful discussions. I thank Mrs. Marcela Ferrer for the preparation of the wheat germ cell-free extracts. I. D. A. is a Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

REFERENCES

1. Schreier, M. H. and Noll, H. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 805-809.
2. Haselkorn, R. and Rothman-Denes, L. F. (1973) Ann. Rev. Biochem. 42, 397-438.

3. Shafritz, D. A., Prichard, P. M., Gilbert, J. M. and Anderson, W. F. (1970) Biochem. Biophys. Res. Commun. 38, 721-727.
4. Marcus, A., Efron, D. and Weeks, D. P. (1974) in Methods in Enzymology (Moldave, K. and Grossman, L., eds.) Vol. 30, pp. 749-754.
5. Efron, D. and Marcus, A. (1973) FEBS Lett. 33, 23-27.
6. Revel, M. (1977) in Molecular Mechanisms of Protein Biosynthesis (Weissbach, H. and Pestka, S., eds.) pp. 245-321, Academic Press, New York.
7. Weissbach, H. and Ochoa, S. (1976) Ann. Rev. Biochem. 45, 191-216.
8. Huang, M. T. and Grollman, A. P. (1972) Mol. Pharmacol. 8, 111-127.
9. Marcus, A., Bewley, J. D. and Weeks, D. P. (1970) Science 167, 1735-1736.
10. Vázquez, D. (1979) in Inhibitors of Protein Biosynthesis, pp. 28-30, Springer Verlag, Berlin.
11. Lodish, H. F., Housman, D. and Jacobsen, M. (1971) Biochemistry 10, 2348-2356.
12. Culp, W. J., McKeehan, W. L. and Hardesty, B. (1969) Proc. Natl. Acad. Sci. U. S. A. 63, 1431-1438.