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AMINOACYL-*t*RNA BINDING AND PEPTIDE CHAIN ELONGATION ON 80s PLANT RIBOSOMES

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Abstract-The various conditions necessary for activity of a transfer system from beans is described. A shift in the Mg^{2+} optimum has been demonstrated which was dependent on deacylated $tRNA^{Phe}$. A GTP dependent enzymatic binding fraction and a fraction involved in polymerization have been partially separated.

INTRODUCTION

THE PROCESS of protein synthesis on the ribosomes can be separated into three sub-processes -initiation, elongation and protein release; the elongation steps are repeated many times. Studies using microbial 70s ribosomes have shown that *m*RNA attaches to the 30s ribosomal sub-unit and f-met *t*RNA enters the peptidyl (donor) site; on association of the 50s sub-unit the amino acid (acceptor) site becomes available, and it is at this point that the steps involving the binding of aminoacyl *t*RNAs and elongation of the peptide chain occur.[1]

In the absence of a natural messenger we have used a poly U-directed system to study the elongation steps of protein synthesis. By using a transfer rather than a complete system, those reactions involving the activation of amino acids prior to peptide bond formation are eliminated from the process, since in the transfer system aminoacyl *t* RNA molecules themselves are added in incubation reactions. However, before the poly U-directed transfer system can be accepted as a suitable model for the investigation of protein chain elongation, it is necessary to ensure that the interaction of the poly U with the ribosome is the same as with natural messengers.

RESULTS

The time courses of incorporation of phenylalanine by the poly U dependent and independent transfer systems, are illustrated in Fig. 1, which shows that in both systems incorporation was completed within 30 min; prolonging the incorporation period up to 3·5 hr did not result in any further increase in the amount of incorporation.

The results of Table 1 show that incorporation is dependent on the presence of microsomes and partially dependent on GTP and enzyme. The temperature optimum of the system was 25°, the pH optimum value 7·8 and the Mg^{2+} optimum 7 mM.

The results in Fig. 2 show that pre-incubation of bean ribosomes, poly U and deacylated *t* RNA prior to a polymerization incubation leads to a shift in the Mg^{2+} optimum from 7 mM to 4 mM. Similar pre-incubations, but without deacylated *t*RNA, have no effect on the optimum, and although the degree of polymerization is then lower, there is a slight increase in incorporation at lower $MgCl_2$ concentration, probably due to a trace of deacylated *t*RNA present in the microsomal preparation. The Mg^{2+} optimum of the transfer system without poly U is 4 mM.

[1] D. BOULTER, Ann. *Rev. PI. Physiol.* 21, *91 (1970).*

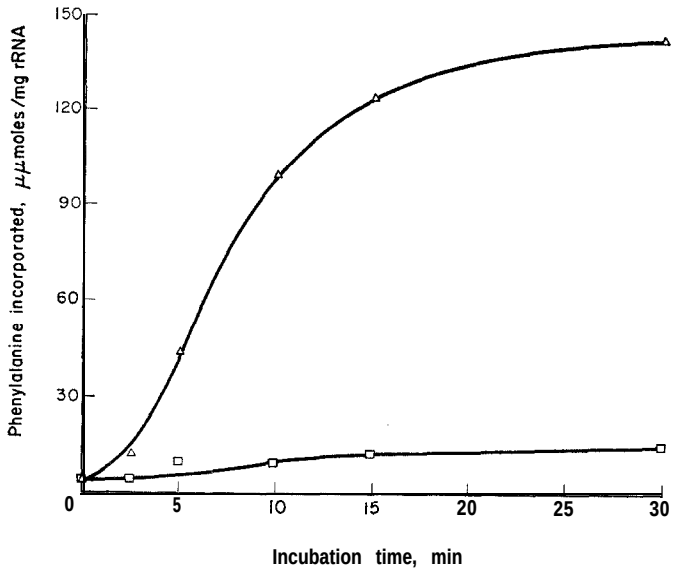

FIG. 1. TIME COURSE OF ENDOGENOUS AND POLY U-DIRECTED INCORPORATION OF PHENYLALANINE. △——△ + poly u; □——□ — poly U.

TABLE 1. CO-FACTOR REQUIREMENTS OF [^{14}C]PHENYLALANINE INCORPORATION USING A TRANSFER SYSTEM

Components	p-mole phenylalanine incorporated/mg *r*RNA
Complete	149
– enzyme	44
– GTP	53
– poly U	12
– Mg^{2+}	6
– microsomes	4

For incubation conditions see Experimental.

Experiments Designed to show the Presence of Binding and Polymerization Enzymes in Bean Enzyme Fractions by use of N-ethyl Maleimide (NEM)

The sulphydryl alkylating reagent, NEM, has been shown to inhibit polyphenylalanine synthesis in the poly U-directed reticulocyte transfer system,[2] due to the inactivation of one or more of the factors present in Fraction II of the reticulocyte transfer factors (i.e. the factors required for peptide synthesis). There was little or no effect of NEM on GTP-dependent enzymatic binding (which required Fraction I or the binding fraction).[2] Reticulocyte fractions will replace those from beans in the bean transfer system, and bean supernatant is virtually as active in the reticulocyte system as is the reticulocyte 40/70

[2] R. D. MOSTELLER, J. M. RAVEL and B. HARDESTY, *Biochem. Biophys. Res. Comm. 24,714* (1966).

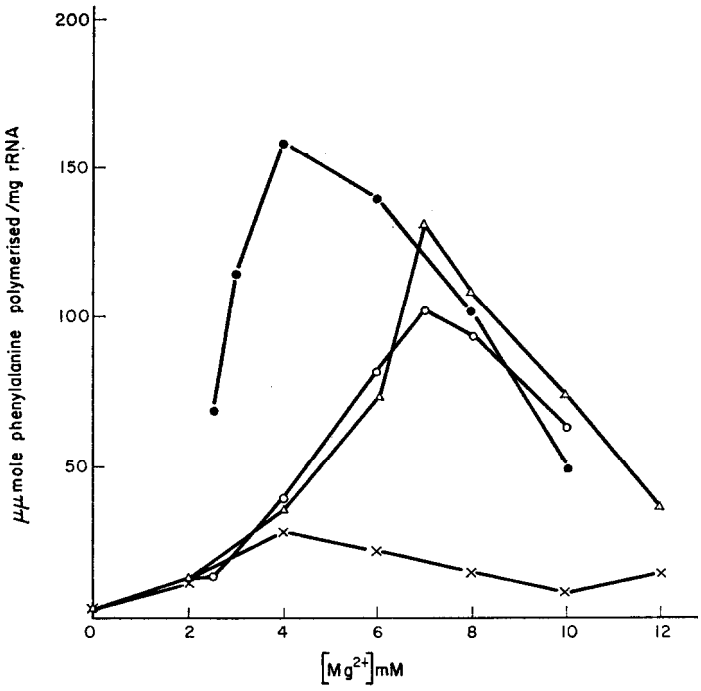

FIG. 2. $[Mg^{2+}]$ OPTIMA FOR POLYPHENYLALANINE SYNTHESIS: EFFECT OF PREINCUBATION WITH DEACYLATED *t* RNA.

Polyphenylalanine synthesis without preincubation was carried out during incubation for 10 min, in a solution containing Mg^{2+} at the concentration indicated, other conditions being as described in Experimental for the bean transfer system.

Preincubation was carried out for 4 min at 37" in 0·25 ml of a solution containing 0·08 M Tris-HCl pH 7.6, 0·07 M KCl, 0·1 M GSH, 100 μg poly U, 50 μg deacylated bean *t*RNA where applicable, 250 μg bean microsomes and 0·01 M $MgCl_2$. After preincubation, the tubes were chilled in crushed ice and the remaining components required for polyphenylalanine synthesis were added to give a final volume of 1·0 ml containing, in addition to the components above, which were maintained at the same concentrations, 0·2 mM GTP, 0·5 mg [^{14}C]phenylalanyl-*t*RNA (23,000 counts/min/mg; Sp. Act. 48 mC/mM), 0·1 ml of bean enzyme, and $MgCl_2$ at the indicated concentration. The tubes were then incubated for 10 min at 37" and assayed for polyphenylalanine synthesis as described previously. [9]

△——△ + poly U, no preincubation; x-x — poly U, no preincubation; ○——○ + poly U, preincubated in the absence of deacylated *t*RNA; ●——● + poly U, preincubated with deacylated *t*RNA.

fraction (unpublished data). It is therefore, quite possible to use bean enzymes in a transfer system otherwise made up of reticulocyte fractions, including NEM treated ribosomes.

Table 2 shows the effect of NEM treatment of enzyme fractions on polymerization of phenylalanine. The results in Table 2 also show that full enzymic activity is regained on the addition of reticulocyte Fraction II to both reticulocyte and bean NEM treated fractions, indicating that NEM treated bean supernatant has Fraction I activity, i.e. contains a binding enzyme. This is largely confirmed by results in Table 3, which show that NEM treated bean supernatant successfully promotes enzymatic binding of phenylalanyl-*t*RNA to ribosomes, and that this binding (which is codon directed), is largely dependent upon the presence of GTP. There is also a small amount of non-enzymatic binding.

TABLE 2. EFFECT OF NEM TREATMENT OF ENZYME FRACTIONS ON POLYMERIZATION OF PHENYLALANINE IN THE RETICULOCYTE TRANSFER SYSTEM

Source of enzyme	counts/min incorporated
None	57
Reticulocyte 40/70 enzyme	13,935
NEM treated reticulocyte 40/70 enzyme	4555
NEM treated reticulocyte 40/70 enzyme + reticulocyte Fraction II	14,130
Bean enzyme	6975
NEM treated bean enzyme	100
NEM treated bean enzyme + reticulocyte Fraction II	7533
Reticulocyte Fraction II alone	2592

For incubation conditions see Experimental.

Paper chromatography of the product collected on Millipore filters, showed that the observed radioactivity recorded in Table 3 was due to enzymatic binding alone, and not to polymerization. From incubations, using reticulocyte Fraction I or NEM-treated bean supernatant, the radioactivity was recovered as free phenylalanine only, and no polymerized phenylalanine was detected.

TABLE 3. BEAN-RETICULOCYTE SYSTEM: SUBSTITUTION OF NEM-TREATED BEAN SUPERNATANT FRACTION FOR RETICULOCYTE FRACTION I IN ENZYMATIC BINDING

Conditions	[^{14}C]Phe tRNA bound (p-moles)
No enzyme fraction	8.2
– GTP	9.5
Reticulocyte Fraction I	23.2
– GTP	9.4
– ribosomes	0·1
NEM bean fraction	23.6
– GTP	11·0
– ribosomes	0·2

The conditions for enzymatic binding are described in Experimental.

The inference drawn from the results in Table 3 was confirmed in experiments using partly purified reticulocyte enzyme Fractions I and II. The data from these experiments presented in Table 4, show conclusively that NEM-treated bean supernatant can substitute for reticulocyte Fraction I. As the bean supernatant fraction contains fractions equivalent to reticulocyte Fractions I and II, an attempt was made to separate these by methods similar to those used by other workers to fractionate the reticulocyte transfer factors.[3]

Bean supernatant was chromatogrammed on a Sepharose column, and the results are illustrated in Fig. 3. This shows that although most of the protein was eluted between Fractions 14 and 17 inclusive, fractions active in polymerization and binding were mainly

[3] W. L. MCKEEHAN and B. HARDESTY, *J. Biol. Chem.* **244**, 4330 *(1969).*

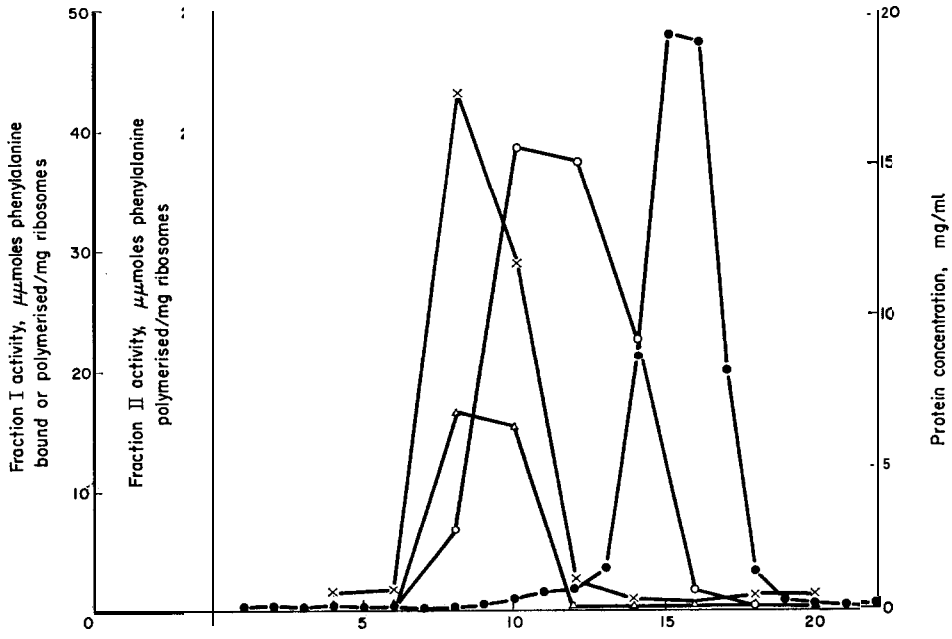

Fig. 3. CHROMATOGRAPHY OF BEAN SUPERNATANT ON SEPHAROSE.
●——● Protein concentration as determined by the method of Warburg and Christian.[14] Fractions 4–20 were each assayed for Fraction I activity in polymerization x-x and Fraction II activity in polymerization O-O by incubating in the reticulocyte transfer system with partially purified reticulocyte Fractions II and I respectively. Fraction I activity in enzymatic binding A-A was determined as in Experimental.

TABLE 4. SUBSTITUTION OF NEM-TREATED BEAN SUPERNATANT FRACTION FOR RETICULOCYTE FRACTION I IN THE RETICULOCYTE TRANSFER SYSTEM

Source of transfer enzymes		
Fractionated Reticulocyte enzyme fractions	NEM-treated bean supematant fraction	[^{14}C]Phenylalanine polymerized (p-moles)
—	—	1.9
F-I	—	1·0
F-II	—	1·6
F-I + F-II	—	37.7
—	+	3.8
F-I	+	5·0
F-II	+	39·9

The enzyme fraction indicated above was assayed for activity in phenylalanine polymerization, in the reticulocyte transfer system.

eluted before this. The first fraction to be eluted was equivalent to reticulocyte Fraction I and showed activity in polymerization (when supplemented with reticulocyte Fraction II) and in the binding assay which was carried out in the presence of NEM to inhibit peptide synthesis. Following this, and only partially separated from it were a number of fractions

which exhibited an activity similar to reticulocyte Fraction II in polymerization. Work is at present in progress to completely separate and characterize these two complementary bean enzyme fractions.

DISCUSSION

The various conditions for optimum activity of the bean transfer system are similar to those described from other sources.

A change has been demonstrated in the Mg^{2+} optimum of phenylalanine polymerization from 7 mM to 4 mM on incubation of the bean ribosomes, poly U and deacylated *t*RNA prior to polymerization in a second incubation. A similar situation has been recorded for the reticulocyte poly U-directed transfer system.[4] Here it was clearly demonstrated that the deacylated *t*RNA species necessary for the shift was $tRNA^{Phe}$.[5] Similar experiments carried out in Dr. Hardesty's laboratory, using reticulocyte components with *Vicia faba* ribosomes, have also shown that it is $tRNA^{Phe}$ which is the active *t*RNA species. An explanation of the role of deacylated *t*RNA in the poly U-directed system has now been given.[6] Lucas-Lenard and Lipmann[7] using a poly U-directed sytem from *Escherichia coli*, showed that N-acetyl phenylalanine *t*RNA and two thermolabile factors were involved in chain initiation. In the presence of these three components, Mg^{2+} optimum for polyphenylalanine incorporation is 4 mM, in contrast to 8 mM in their absence.

These studies indicate that the poly U-directed system is a satisfactory model for chain elongation studies in protein syntheiss. Using the system, thermolabile factors have been isolated, one responsible for GTP dependent enzymatic binding of Phe *t*RNA, the other involved in peptide synthesis. Similar factors have been isolated from microbes, mammals and wheat germ.[1,8]

EXPERIMENTAL

Biological materials and chemicals. The biological materials, chemicals, buffers and reagents were as previously described.[9]

***Extraction of t**RNA.* *t*RNA was prepared from developing bean cotyledons by the method of Mosteller *et al.*,[10] as modified in Payne *et al.*[9]

***Preparation of charged transfer RNA,* *t* RNA** prepared from 60-day-old developing bean seeds was charged with [^{14}C]phenylalanine and 19, [^{12}C]amino acids by the method of Ravel *et al.,"* except that the *t*RNA was incubated at 25" for 30 min, the '40/70 ammonium sulphate fraction' was replaced by the 105,000 *g* supernatant fraction from 60 day seeds and final chromatography on Sephadex G-25 was omitted.

The bean transfer system. Incubations contained the following in μmoles/ml incubation: Tris-HCl buffer, pH 7-6 at 25". 60: $MgCl_2$, 7: KCl, 70: GSH, 10: GTP, 0·2: also per ml incubation: poly U, 0·4 mg; *t*RNA previously charged with [^{14}C]phenylalanine (specific activity 44·2 mc/m-mole) and 19 × ^{12}C amino acids, 0·4 mg (21,000 counts/min/mg); enzyme, 0·06 ml; and microsomes, 1 mg as determined by E^{256}_{1cm}. The amount of incorporation after 20 min was measured on Millipore filters as described previously.[9]

The reticulocyte transfer system. The various components and incubation conditions were as described in Mosteller *et al.*,[10] Ravel *et al."* and Lin *et al.*[12]

[4] R. D. MOSTELLER, W. J. CULP and B. HARDESTY, *J. Biol. Chem.* 243, 6343 (1968).
[5] W. J. CULP, R. D. MOSTELLER and B. HARDESTY, *Arch. Biochem. Biophys.* 125, 658 (1968).
[6] M. H. SCHREIR and H. NOLL, *Nature, Lond. 227. 128 (1970).*
[7] J. LUCAS-LENARD and F. LIPMANN, *Proc. Natl. Acad. Sci.* **57**, 1050 (1967).
[8] A. B. LEGOCKI and A. MARCUS. *J. Biol. Chem. 245. 2814* (1970).
[9] E. S. PAYNE, D. BOULTER, A. BROWNRIGG, D. LONSDALE, A. YARWOOD and J. N. YARWOOD, *Phytochem,* 10, 2293 (1971).
[10] R. D. MOSTELLER, W. J. CULP and B. HARDESTY, *Proc. Nat/. Acad. Sci. 57,* 1817 (1967).
[11] J. M. RAVEL, R. D. MOSTELLER and B. HARDESTY, *Proc. Natl. Acad. Sci.* 56, 701 (1966).
[12] S. LIN, R. D. MOSTELLER and B. HARDESTY, *J. Mol.* Biol. 21, 51 (1966).

Enzymatic binding assay. [^{14}C]Phenylalanyl-*t*RNA enzymatically bound to reticulocyte NaF DOC ribosomes was determined by the following procedure, based on that of Nirenberg and Leder.[13] 0·5 ml of a solution containing 60 mM Tris-HCl pH 7·5, 70 mM KCl, 4 mM $MgCl_2$, 0·1 mM GTP, 0·1 mg poly U, 0·5 mg reticulocyte NaF, DOC ribosomes and [^{14}C]phenylalanyl-*t*RNA together with the enzyme fraction indicated was incubated for 15 min at 37". The reaction was terminated by diluting lo-fold with cold buffer containing 0·05 M Tris-HCl pH 7.5, 0·05 M KCl and 8 mM $MgCl_2$, and immediately filtering through Millipore filters, which were then washed with a further 15 ml of buffer. The filters were dried and counted as described previously.[9] Values for non-enzymatic binding were routinely determined and the figures for enzymatic binding corrected. All enzymatic binding assays were carried out either in the presence of NEM to inhibit peptide synthesis, or using NEM treated enzyme fractions.

Chromatography of the radioactive product of enzymatic binding. Millipore filters from enzymatic binding assays were placed in tubes containing 1·0 ml of 2·5 M NH_4OH and incubated with periodicvigorous shaking for 30 min at 37". The filters were removed from the tubes, dried and counted to ensure that all radioactivity had been removed. The tube contents were dried *in vacuo*, the residues redissolved in 0·1 ml H_2O and subjected to ascending (chromatography on Whatman No. 1 chromatography paper using the solvent *n*-BuOH–H_2O–HOAC (4:1:1 by vol.). After development, the chromatograms were dried, cut into strips and counted for radioactivity.

Under the conditions, used free phenylalanine should have an R_f value of 0·5–0·6 while phenylalanylphenylalanine should have an R_f of 0·85.[14]

Fractionation of bean high-speed supernatant by gel-filtration on sepharose 4B. The method used was essentially that of McKeehan and Hardesty.[3] A 2 x 20 cm column of Sepharose 4B was equilibrated with a buffer containing 0·01 M Tris-HCl, pH 7.5, 0·25 M KCl, 5 mM mercaptoethanol, 0·2 mM EDTA, at 4". 2·0 ml of *Vicia faba* high-speed supernatant was saturated with sucrose and applied to the top of the column under a 5 cm column of buffer.

The column was eluted with the same buffer at a flow rate of 38 ml/hr, the first 22 ml of the eluate, approximately equivalent to the void volume of the column, was discarded and then fractions of approximately 3·2 ml were collected. The fractions were assayed for protein by the method of Warburg and Christian,[15] and for activity in promoting enzymatic binding and polymerization of phenylalanine as described above. The enzyme assays were carried out without correcting for the K^+ in the column fractions.

Reticulocyte transfer factors. Partially purified reticulocyte transfer factors were kindly supplied by Dr. Boyd Hardesty (see ***Acknowledgements).*** Fraction I was prepared by Sepharose chromagraphy,[3] and was treated with NEM to reduce contaminating Fraction II activity. Fraction II had been further purified by DEAE-cellulose chromatography and contained no contaminating Fraction I activity.

Preparation of NE&f-treated components. NEM treatment of NaF treated DOC washed reticulocyte ribosomes was as described by Mosteller et al.[10] For the preparation of NEM treated enzyme preparations 0·05 ml of 0·1 M NEM was added to 0·5 ml 40/70 reticulocyte fraction," or to 0·20 ml bean supematant to which had previously been added 0·25 ml 0·01 M Tris (pH 7.5); 0002 M GSH, 0·001 M EDTA. The oreuarations were allowed to stand for 10 min at 0". and then 0·02 ml 0·05 M GSH was added to each and allowed to stand for 5 min at 0" to react with the excess NEM.

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[13] M. NIRENBERG and P. LEDER, ***Science 145,*** 1399 (1964).
[14] S. LIN, W. L. MCKEEHAN, W. J. CULP and B. HARDESTY, ***J. Biol.*** Chem. 2444340 (1969)
[15] 0. WARBURG and W. CHRISTIAN, ***Biochem. Zeitschrift 310, 384 (1942).***