THE REQUIREMENT FOR tRNA FOR THE SHIFT IN THE OPTIMUM Mg^{++} CONCENTRATION DURING THE SYNTHESIS OF POLYPHENYLALANINE

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Numerous reports from many laboratories present data that have been interpreted to indicate the involvement of tRNA in regulation of protein synthesis at the translation level. The initiation of peptides on ribosomes is of particular interest in that the reactions involved may provide the site for this type of control. Here we present evidence that indicates a requirement for very small amounts of tRNA in reactions that preceed the GTP-dependent step of peptide bond formation. Deacylated tRNA is at least as active for these steps as phenylalanyl-tRNA or N-acetylphenylalanyl-tRNA. In apparent contrast to poly U directed systems derived from bacteria, the reactions involved do not lead to efficient incorporation of N-acetylphenylalanine into the N-terminal position of polyphenylalanine. These observations may reflect basic differences at the level of the initiation of peptides between bacterial and mammalian systems.

MATERIALS AND METHODS

The conditions for assay of globin or polyphenylalanine synthesis have been described (Ravel, Mosteller and Hardesty, 1966; Mosteller, Culp and Hardesty, 1967). Also described were the materials and procedures for preparations of ribosomes, enzyme fraction and rabbit liver tRNA. The procedure used for deacylation produces 97% hydrolysis of phe-tRNA[1] and greater than 80% hydrolysis of acetylphe-tRNA.

[1] Abbreviations used: phe-tRNA - phenylalanyl-tRNA
acetylphe-tRNA - N-acetylphenylalanyl-tRNA
$tRNA_{phe}$ - species of tRNA that can be charged with phenylalanine
Mg^{++} shift - the shift in the optimum Mg^{++} concentration for polyphenylalanine synthesis from 8 mM without preincubation to 4 mM following preincubation

N-acetyl-^{3}H-phenylalanyl-tRNA was prepared by two different methods (Haenni and Chapeville, 1966; de Groot, Lapidot, Panet and Wolman, 1966). No significant difference in acetylphe-tRNA prepared by the two procedures was detected in the experiments reported here.

RESULTS AND DISCUSSION

As shown in Figure 1, the incorporation of valine into globin peptides in the reticulocyte transfer system has an optimum Mg^{++} concentration near 3 mM. The poly U directed synthesis of polyphenylalanine has an optimum Mg^{++} concentration near 8 mM. Preincubation of the poly U directed system re-

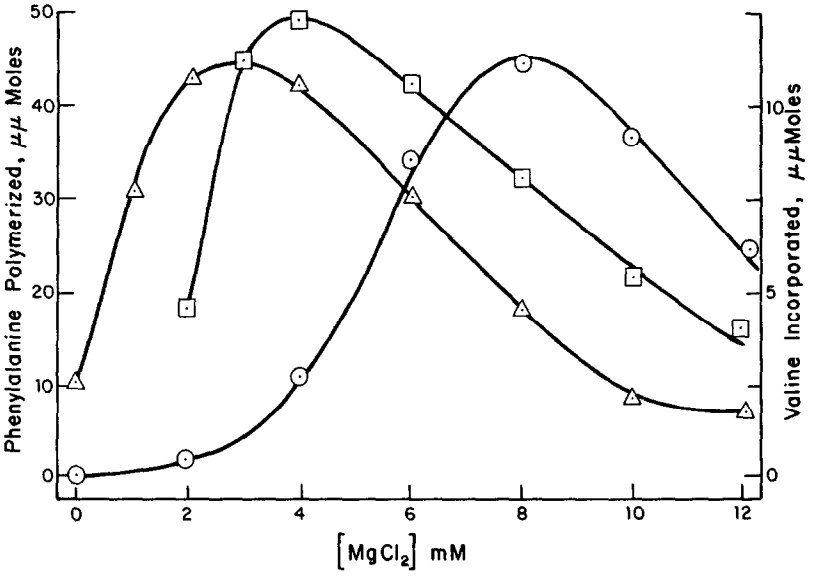

Figure 1. Mg^{++} optima for globin and polyphenylalanine synthesis. The experimental design is explained in the text. Incubation for globin synthesis was at 37° for 5 minutes with 0.5 ml of a reaction mixture containing 0.06 M Tris, pH 7.5, 0.07 M KCl, 0.2 mM GTP, 0.01 M reduced glutathione, 500 µg of ribosomes, 100 µg of protein from Fraction I + II, 100 µg (about 5000 cpm) of tRNA charged with ^{14}C-valine (20 C/mole) and 19 other amino acids, and $MgCl_2$ as indicated.

Incubation (5 minutes at 37°) for polyphenylalanine synthesis contained 100 µg of poly U, ^{14}C-phe-tRNA (2000 cpm, 20 C/mole phe) in place of val-tRNA, and 250 µg of ribosomes in a final volume of 1.0 ml. Preincubation was at 37° for 8 minutes of a 0.25 ml reaction mixture containing 0.06 M Tris, pH 7.5, 0.07 M KCl, 0.008 M $MgCl_2$, 0.01 M reduced glutathione, 250 µg of ribosomes, 100 µg poly U, and 2 µg of deacylated tRNA.

Δ—Δ—Δ, valine into globin peptides

O—O—O, polyphenylalanine without preincubation

□—□—□, polyphenylalanine following preincubation

sults in a shift in the Mg^{++} optimum to near that for incorporation into globin, to about 4 mM Mg^{++} concentration in these experiments. Preincubation at 3,4 or 8 mM Mg^{++} of the system for the incorporation of valine into globin does not result in a change in the optimum Mg^{++} concentration, although the amount of valine incorporated is generally reduced. These optima, 8 mM and 4 mM, are very similar to optima observed for polyphenylalanine synthesis in a transfer system derived from bacteria (Lucas-Lenard and Lipmann, 1967).

In the experiments reported here, the Mg^{++} shift for polyphenylalanine synthesis was produced by preincubating ribosomes, poly U and 2 µg of deacylated tRNA together in a reaction mixture containing 8 mM Mg^{++}. After preincubation, the samples were chilled and then diluted to the indicated final concentration of Mg^{++} by the addition of a soluble enzyme fraction, GTP, and 100 µg of ^{14}C-phe-tRNA, and the other components required for polymerization. Polyphenylalanine was formed during a subsequent incubation. The system used in these studies is highly dependent upon added GTP and requires the addition of at least two soluble components for the synthesis of peptide bonds. No detectable diphenylalanine or larger polypeptides were formed during the preincubation.

The Mg^{++} shift is dependent upon preincubation together of ribosomes, poly U and tRNA. The requirement for tRNA to bring about the Mg^{++} shift during preincubation is depicted in Figure 2. The experiments represented by this figure were performed in a manner similar to those described for Figure 1, except that all tubes were incubated at 4 mM Mg^{++}. Preincubation mixtures contained the indicated amounts of phe-tRNA, acetylphe-tRNA or deacylated tRNA. Experiments performed with a number of preparations of tRNA generally show greater amounts of polymerization at 4 mM Mg^{++} following preincubation with deacylated tRNA than with acetylphe-tRNA or phe-tRNA. The nature and significance of the apparent different is not clear. Near maximum polyphenylalanine synthesis occurred with about 2 µg of any one of the three forms of tRNA. This is a surprisingly small amount of tRNA for apparent saturation of the system producing the shift in optimum Mg^{++} concentration. Assuming that the preparation contains only tRNA of a weight of about 23,000 (Cantoni and Richards, 1966) and that the molecular weight of the reticulocyte ribosomes is 4.0×10^6 daltons (Ts'o and Vinograd, 1961), it may be calculated that apparent saturation occurs at a ratio of 1.3 molecules of the unfractionated tRNA per ribosome.

Lucas-Lenard and Lipmann (1967) have reported conditions for a system derived from E. coli in which maximum polyphenylalanine synthesis was obtained at a relatively low concentration of Mg^{++} without preincubation. In this case, maximum polymerization at 4 mM Mg^{++} instead of 8 mM Mg^{++} was dependent

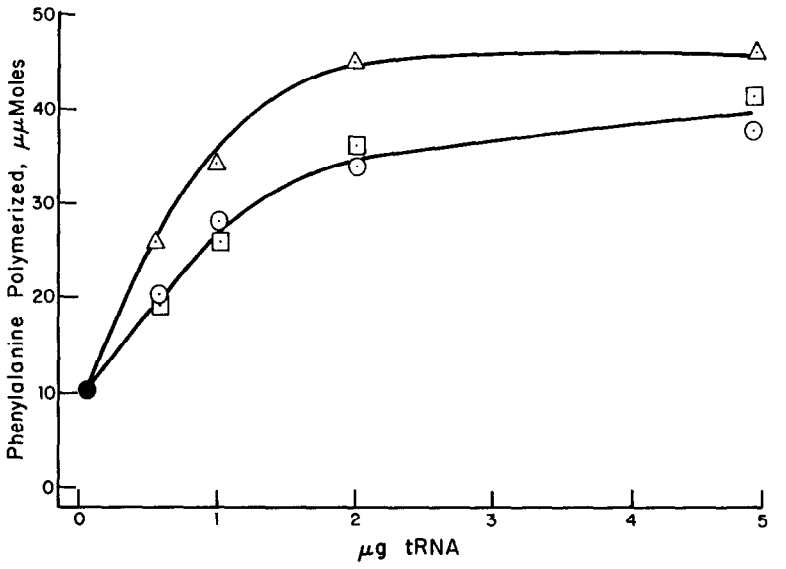

Figure 2. Requirement for tRNA to bring about the Mg^{++} shift during preincubation. The experimental design is similar to that described for Figure 1. The indicated amounts of tRNA were present during preincubation.

Δ—Δ—Δ, deacylated tRNA in the preincubation

O—O—O, phe-tRNA in the preincubation

□—□—□, acetylphe-tRNA in the preincubation

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on the chain initiation factors, f_1 and f_2, and upon acetylphe-tRNA. Results were interpreted to indicate the incorporation of N-acetylphenylalanine as the N-terminal amino acid of polyphenylalanine. This conclusion was in agreement with earlier work by Haenni and Chapeville (1966).

It has not been possible to demonstrate a similar effect of chain initiation factors or acetylphe-tRNA in lowering the optimum Mg^{++} concentration without preincubation in the reticulocyte system. A chain initiation factor(s), present in most preparations of ribosomes, is involved in the initiation of polyphenylalanine (Mosteller, Culp and Hardesty, 1967) and for the de novo synthesis of hemoglobin (Miller, Hamada, Yang, Cohen and Schweet, 1967), but it appears to function at a step following the reactions that are involved in the Mg^{++} shift. Preparations of ribosomes rich in the factor and ammonium chloride washes of these ribosomes have failed to lower the optimum Mg^{++} concentration to 4 mM without preincubation as reported for the E. coli system. Representative data are presented in Table I. For these experiments, numerous forms of washed and unwashed ribosomes in varying combinations with various crude and partially resolved fractions containing enzymes derived from the

soluble fraction and from NH_4Cl washes of the ribosomes were incubated with the three types of tRNA.

It can be effectively argued that these negative data for the Mg^{++} shift reflect unstable chain initiation factors or other basic deficiencies in the reticulocyte transfer system. Interpretation of the data is rendered even more difficult by the dependence of the optimum Mg^{++} concentration and rate of synthesis on the amount of protein and nucleic acid present in the system. Conclusive evidence against the existence of the factors is lacking, however under the conditions used here, deacylated tRNA is active and acetylphe-tRNA is not required for the shift in optimum Mg^{++} concentration that is brought about by preincubation. Further, under these conditions very little if any N-acetylphenylalanine from acetylphe-tRNA added during preincubation is incorporated into the N-terminal position of polyphenylalanine. Typical results reflecting the low incorporation of N-acetylphenylalanine from 2 μg of tRNA added before preincubation are also presented in Table I. In addition, similar experiments performed with phe-tRNA added during preincubation indicated that the phenylalanine residue of this tRNA was not preferentially incorporated into polyphenylalanine. N-terminal analyses of polyphenylalanine formed in the presence of acetylphe-tRNA indicate phenylalanine with a free amino group as the N-terminal amino acid and indicate that it is derived from phe-tRNA.

The rather surprising result that phenylalanine or N-acetylphenylalanine from tRNA required for the Mg^{++} shift is not preferentially incorporated into the N-terminal position of polyphenylalanine might be explained by a rapid deacylation of this tRNA. The results of experiments designed to test this possibility indicated that less than 10% of the acetylphe-tRNA or phe-tRNA added before preincubation was hydrolyzed as the Mg^{++} shift occurred during preincubation. The lack of incorporation of N-acetylphenylalanine into polyphenylalanine might reflect the involvement of a species of tRNA other than $tRNA_{phe}$ in these initial reactions. Evidence to be presented elsewhere (Culp, Mosteller and Hardesty, 1968) indicate a high correlation between the amount of $tRNA_{phe}$ present in fractionated preparations of tRNA and the ability of these fractions to promote the Mg^{++} shift.

The apparent dissimilarity in the requirements for the Mg^{++} shift and incorporation of N-acetylphenylalanine into polyphenylalanine may indicate basic differences in reactions associated with the initiation of peptides in the E. coli and rabbit reticulocyte systems. These differences may reflect the form of messenger RNA and the mechanism by which the initiating codon is utilized to direct the integration of messenger RNA into ribosome complexes in bacteria and higher organisms.

Table I. Requirement for preincubation to obtain the Mg^{++} shift and failure to incorporate N-acetylphenylalanine.

Conditions	Phenylalanine as Polyphenylalanine μμmoles	Incorporation of N-acetylphenylalanine cpm of ^3H	μμmoles
Preincubation			
without acetylphe-tRNA	10	-	-
with acetylphe-tRNA*	42	17	< 0.05
No preincubation			
without acetylphe-tRNA	9	-	-
with acetylphe-tRNA*	9	15	< 0.05
with acetylphe-tRNA* (crude systems)	4-11	11-19	< 0.05

*About 600 cpm (910 C/mole phe) of N-acetyl-^{3}H-phe-tRNA in 2.0 μg of tRNA. Conditions for preincubation and incubation were described in Figure 1. All preincubation mixtures contained 8 mM Mg^{++}. All incubation mixtures contained 4 mM Mg^{++}. Amounts of up to 20 μg of N-acetylphe-tRNA did not increase polyphenylalanine synthesis without preincubation.

Crude systems - In addition to the regular components, the crude incubation mixtures contained 10-400 μg of soluble enzymes from the following sources in various combinations with 0.5 mg of ribosomes of the type listed below:

Enzyme fraction - supernatant from fresh reticulocyte lysate after high speed centrifugation, 40-70% ammonium sulfate fraction of the high speed supernatant or subfractions of this ammonium sulfate fraction, and protein from a 1 M NH_4Cl wash of the ribosomes.

Ribosomes - deoxycholate washed ribosomes, 0.5 or 1.0 M NH_4Cl washed ribosomes, unwashed ribosomes from reticulocytes incubated with NaF, regular reticulocyte ribosomes containing polysomes and nascent globin chains. Values of polyphenylalanine synthesis with ribosomes capable of appreciable incorporation of phenylalanine into globin peptides were corrected on the basis of valine incorporation assuming an incorporation ratio of valine to phenylalanine into globin of 2.

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