THE INITIATION OF POLYPHENYLALANINE SYNTHESIS WITH N-ACETYLPHENYLALANYL-tRNA*

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Formylation of Met-tRNA modifies it into a molecule that resembles peptidyl-tRNA. Although this modification is not the only factor involved in making fMet-tRNA an effective initiator of protein synthesis, it appears to play an important role.1-3 Like fMet-tRNA, the dipeptidyl-tRNA of phenylalanine and N-acetylPhe-tRNA stimulate polypeptide synthesis at low magnesium ion concentrations.5, 4, 5 The acetylated phenylalanine is incorporated into the N-terminal end of polyphenylalanine from N-acetylPhe-tRNA.6 In addition, the ribosomal factors required for initiating synthesis with fMet-tRNA promote the binding of N-acetylPhe-tRNA to ribosomes and are essential for the stimulation of polyphenylalanine synthesis by the acetylated Phe-tRNA.4, 7

We reported previously that a factor in the unfractionated supernatant of Escherichia coli extract was essential for the initiation of polypeptide synthesis with fMet-tRNA and N-acetylPhe-tRNA.8 Since several initiation factors had already been described7, 9, 10 and we were interested in determining the exact relation of our factor to those observed by the other investigators, we undertook a more thorough characterization of this component. The results of these studies, reported orally in part,11 are presented in this communication.

Materials and Methods.—Preparation of ribosomes and supernatant: E. coli cells were ground with alumina in 2 vol of medium containing 0.01 M imidazole, pH 7.4, 0.01 M MgCl₂, 0.04 M KCl, and 0.001 M 2-mercaptoethanol (standard buffer). DNase was added to a concentration of 1 μg/ml and the suspension was centrifuged for 15 min at 30,000 × g. The resulting S-30 extract was then centrifuged for 3½ hr at 50,000 rpm in a Beckman Ti 50 rotor. The supernatant was used in the preparation of protamine sulfate-treated supernatant. The ribosomal pellet was resuspended in standard buffer at a concentration of 20-30 mg of ribosomes/ml and frozen in small aliquots in a dry ice-acetone mixture. The ribosomes and protamine sulfate-treated supernatant were stored at −60° and were not refrozen after having been thawed. The supernatant used in Figures 1 and 2 was prepared by centrifuging the S-30 extract for 6 hr at 35,000 rpm in an International SB 206 rotor (swinging bucket rotor with maximum centrifugal force of 206,000 × g). The top two thirds of the resulting supernatant was removed and dialyzed overnight in the standard buffer before use. Ribosome concentration was determined from optical density measurements at 260 mμ, with the assumption that 14.4 units are equivalent to 1 mg of ribosomes per millilitre.

Preparation of factor: Unwashed ribosomes, at concentrations of 2.5-20 mg/ml, were kept at 0° for 30-60 min in standard buffer with 0.02 M putrescine and 4 × 10⁻⁴ M spermidine before 0.2-ml aliquots were layered on standard 5-ml sucrose gradients containing polyanines. The gradients were centrifuged for 80-300 min at 65,000 rpm in a Beckman SW 65 rotor. The factor was located by assaying 0.05 ml of gradient fraction in a reaction mixture containing 0.004 M MgCl₂, 20-50 μg of low-factor ribosomes (low in factor and 30S subunits, prepared by centrifuging ribosomes through a 5% sucrose solution with 0.05 M imidazole, pH 7.4. 0.006 M MgCl₂, 0.16 M NH₄Cl, 0.02 M putrescine, 4 × 10⁻⁴ M spermidine, and 0.001 M 2-mercaptoethanol) and other components of the standard assay (see below). Incubation was for 4 min at 37°. Active fractions were pooled and used immediately or kept at 0°.
Other preparations: Protamine sulfate-treated supernatant, Phe-tRNA, and N-acetylPhe-tRNA were prepared as described previously. 3,5

Assay procedure: The standard assay measured incorporation of N-acetyl-14C-phenylalanine into hot TCA-precipitable material. The reaction mixture contained 0.05 M imidazole, pH 7.4, 0.08 M NH4Cl, 0.012 M 2-mercaptoethanol, 10–25 µg of poly U, 0.001 M GTP, 0.01–0.02 ml of protamine sulfate-treated supernatant, 60 µg of unlabeled Phe-tRNA, and 30 µg of N-acetyl-14C-Phe-tRNA of specific activity 356 µc/µmole, in a final volume of 0.25 ml. MgCl2, ribosomes, and factor were added as indicated. The reaction was stopped with 3 ml of 5% TCA, the tube was heated for 10 min at 90°, and the precipitate was washed on a Millipore filter. Radioactivity was measured in a Nuclear-Chicago gas-flow counter.

Sucrose density gradients: All gradients used were either 5- or 12-ml linear gradients containing 5–20% sucrose, 0.01 M imidazole, pH 7.4, 0.006 M MgCl2, 0.08 M NH4Cl, 0.001 M 2-mercaptoethanol, and, where indicated, 0.02 M putrescine and 4 × 10−4 M spermidine. Fractions were collected by piercing the bottom of the tubes or by siphoning with a fine tubing. In all cases, alternate fractions of 0.05 ml and 0.1-0.5 ml were collected. The 0.05-ml fractions were diluted 20 times and their optical density at 260 mµ was determined.

Materials: E. coli B cells (1/4 log or 1/2 log) were obtained frozen from Grain Processing Corporation, Muscatine, Iowa, and kept at −60° until used. Poly U was a product of Miles Chemicals, Elkhart, Indiana.

Results.—The stimulation of polyphenylalanine synthesis by N-acetylPhetRNA at varying levels of magnesium ions was strikingly similar to that of viral protein synthesis by fMet-tRNA when initial rates of synthesis were measured (Fig. 1). N-acetylPhe-tRNA strongly stimulated phenylalanine incorporation at low concentrations of magnesium ions, giving rise to a peak of incorporation at a magnesium concentration lower than the optimum observed without the acetylated Phe-tRNA. Unlike the results of earlier studies, maximum phenylalanine incorporation with N-acetylPhetRNA was much greater than the maximum seen at a higher concentration of magnesium in the absence of the acetylated aminoacyl-tRNA. 3,4

The unfractionated supernatant, previously reported to contain a factor es-
sential for the initiation of polypeptide synthesis with fMet-tRNA and N-acetyl-Phe-tRNA, was found to contain considerable amounts of ribosomes, especially 30S subunits, when analyzed by sucrose density gradient centrifugation (Fig. 2). The ability to stimulate N-acetylphenylalanine incorporation was found in the fractions containing the 30S particles and in the upper part of the gradient preceding slightly the rise in optical density. The latter peak of activity was reduced considerably when spermidine and putrescine were not included in the sucrose gradient.

The factor appeared to be absent from the 70S ribosomes separated from freshly prepared S-30 extract of E. coli by sucrose density gradient centrifugation (Fig. 3). Fractions collected from the gradient were assayed for their capacity for N-acetylphenylalanine incorporation with and without the addition of factor-containing 30S ribosomes. Incorporation was almost negligible in the absence of

Fig. 2.—Presence of factor in supernatant. Supernatant (1/10 ml) obtained by centrifuging S-30 extract in the International SB 206 rotor (see Materials and Methods) was layered on a 5-ml sucrose gradient containing 0.02 M putrescine and 4 × 10⁻⁴ M spermidine and centrifuged for 110 min at 39,000 rpm in a Spinco SW 39 rotor. The reaction mixture contained 0.10 ml of gradient fraction, 0.02 M putrescine, 4 × 10⁻⁴ M spermidine, 0.004 M MgCl₂, 70 μg of preincubated low-factor ribosomes (see Materials and Methods), and the other components of the standard assay. Preincubation of the ribosomes was carried out for 10 min at 37° in a medium with MgCl₂, NH₄Cl, and polyamines at levels used in the assay. Incubation was for 2 min at 25°. Curve with broken line represents incorporation.

Fig. 3.—Effect of 30S ribosomes on the incorporation of N-acetylphenylalanine by 70S and 50S ribosomes. Freshly prepared S-30 extract (1/10 ml), diluted to about 33 OD 260 mg units/ml, was layered on a 12-ml sucrose gradient and centrifuged for 3 hr at 35,000 rpm in an International SB 206 rotor. The 30S ribosomes were obtained by the same procedure, except that the S-30 extract used was about three times more concentrated. The reaction mixture contained 0.1 ml of gradient fraction, 0.05 ml of 30S ribosomes where indicated, 0.006 M MgCl₂, and other components of the standard assay. Incubation was for 2 min at 37°. Lower curve with broken line shows incorporation in the absence of the 30S fraction.
the 30S particles, whereas significant amounts of N-acetylphenylalanine were incorporated by the 70S and 50S ribosomes with the addition of the 30S fraction. The results are in marked contrast to those for polyphenylalanine synthesis without N-acetylPhe-tRNA, which occurs readily on 70S ribosomes even when factor or 30S particles are not added. Dissociation of 70S ribosomes by dialysis in a medium containing $10^{-4} M$ Mg$^{++}$ yielded 30S subunits deficient in factor activity.

To study the effect of polyamines on the release of factor from the 30S particles, ribosomes with bound factor were isolated and fractionated by sucrose density gradient centrifugation. Factor activity in the fractions from the gradient was assayed by measuring the stimulation of N-acetylphenylalanine incorporation in a system containing ribosomes low in factor (Fig. 4). When spermidine and putrescine were excluded from the sucrose gradient, only a small part of the factor initially associated with the ribosomes was released and appeared in the upper region of the gradient. But when the ribosomes were centrifuged through a gradient containing polyamines, most of the factor was released from the 30S particles. Spermidine alone was not effective at $4 \times 10^{-4} M$ but caused the release of factor from the ribosomes at $2 \times 10^{-4} M$. Putrescine at $0.02 M$ brought about the release of factor although not as efficiently as in combination with $4 \times 10^{-4} M$ spermidine. It should be pointed out here that in the experiment represented by Figure 2, the assay for N-acetylphenylalanine incorporation was carried out at polyamine concentrations which release the factor from the 30S ribosomes.

**Fig. 4.**—Release of factor from 30S ribosomes. Unwashed ribosomes (83 $\mu g$) in 0.2 ml of standard buffer were layered on each of two 5-ml gradients, one gradient without polyamines (optical density profile shown on left panel) and one with $0.02 M$ putrescine and $4 \times 10^{-4} M$ spermidine. The gradients were then centrifuged for 45 min at 65,000 rpm in a Beckman SW 65 rotor. In the assay for factor activity, each tube contained 0.07 ml of gradient fraction, 0.006 $M$ MgCl$_2$, 50 $\mu g$ of low-factor ribosomes, $5.6 \times 10^{-3} M$ putrescine, $1.1 \times 10^{-4} M$ spermidine, and other components of the standard assay system. The reaction mixtures were preincubated without the unlabeled Phe-tRNA for 6 min at 37° and then incubated for 2 min at 37° after the addition of the unlabeled Phe-tRNA. Similar results were obtained without pre-incubation at $0.004 M$ MgCl$_2$ by a 4-min incubation at 37°. Radioactivity is represented by curve with broken line.
The requirement of factor for N-acetylphenylalanine incorporation by 70S ribosomes was demonstrated with ribosome-free factor (Fig. 5). As seen earlier, the 70S ribosomes were essentially inactive for N-acetylphenylalanine incorporation. The ribosomes became active only upon addition of factor. The results show that the 70S ribosomes can utilize the factor and initiate synthesis with N-acetylPhe-tRNA. The observation, however, provides no clue as to whether the ribosomes undergo dissociation in initiating synthesis.

Although the incorporation of N-acetylphenylalanine, i.e., the initiation of polyphenylalanine synthesis with N-acetylPhe-tRNA, was stimulated by the factor, the synthesis of polyphenylalanine in the absence of the acetylated Phe-tRNA was not (Table 1). N-acetylphenylalanine incorporation was enhanced at 0.004 M as well as at 0.01 M MgCl₂. The factor was inactivated by heating for 2 min of 90°. The binding of N-acetylPhe-tRNA to ribosomes was stimulated by the factor (Table 2). GTP was essential for this stimulation.

To characterize the factor further, an extract of the ribosomes containing the factor was chromatographed on DEAE-Sephadex by the procedure of Ohta et al.¹² The elution pattern obtained suggested that our factor preparation contains factor f2 of Salas et al.⁷ (Fig. 6). Activity was eluted from the resin at a salt concentration which has been shown to elute factor f2.¹² Since the chromatographic procedure separates factors f1 and f2,¹² the results imply that factor f1 was present in the ribosomes used for the assay.

![Figure 5](image)

**Fig. 5.**—Stimulation of N-acetylphenylalanine incorporation by factor separated from ribosomes. Fresh S-30 extract was layered on 5-ml gradient without polyamines and centrifuged for 45 min at 65,000 rpm in a Beckman SW 65 rotor. N-acetylphenylalanine incorporation by 0.1 ml of gradient fraction was measured with and without 0.05 ml of ribosome-free factor (see Materials and Methods) in the standard assay mixture containing 0.004 M MgCl₂. Incubation was for 3 min at 37°. Lower curve with broken line represents incorporation without the addition of factor.

![Figure 6](image)

**Fig. 6.**—Two ml of factor preparation (see Materials and Methods), containing 18 OD 280 mu units, was dialyzed against a solution with 0.02 M Tris-HCl, pH 7.4, 0.01 M NH₄Cl, 0.001 M EDTA, and 0.003 M 2-mercaptoethanol and applied to a DEAE-Sephadex A-50 column (1 × 15 cm) equilibrated in the same buffer solution. After the column was washed with 20 ml of the buffer solution, the absorbed material was eluted stepwise with 20-ml portions of buffer solution in which the NH₄Cl was increased to 0.15, 0.30, and 1 M. Factor activity in indicated fractions was assayed in a system with 65 µg of low-factor ribosomes, 0.007 M MgCl₂, 0.12 M NH₄Cl, 0.10 ml of gradient fraction, and other components of the standard assay. Incubation was for 4 min at 37°. Incorporation of N-acetylphenylalanine is represented by the rectangular columns.
TABLE 1. Effect of factor on N-acetylphenylalanine and phenylalanine incorporation.

<table>
<thead>
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<th>Expt.</th>
<th>Mg++</th>
<th>Additions</th>
<th>Cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.004 M</td>
<td>N-acetyl-14C-Phe-tRNA</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>162</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>+ factor</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>0.01 M</td>
<td>&quot;</td>
<td>52</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>+ factor</td>
<td>180</td>
</tr>
<tr>
<td>6</td>
<td>0.004 M</td>
<td>14C-Phe-tRNA</td>
<td>428</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>+ factor</td>
<td>421</td>
</tr>
<tr>
<td>8</td>
<td>0.01 M</td>
<td>&quot;</td>
<td>2,409</td>
</tr>
<tr>
<td>9</td>
<td>&quot;</td>
<td>+ factor</td>
<td>2,562</td>
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For assay of factor activity, 31 μg of low-factor ribosomes were incubated in the standard reaction mixture with 0.05 ml of factor for 4 min at 37°. The factor was heated for 2 min at 90°, where indicated. The assay for phenylalanine incorporation was similar to that for N-acetylphenylalanine incorporation, except that 60 μg of 14C-Phe-tRNA of specific activity 356 μc/μmole was the only aminoacyl-tRNA present and incubation was for 1 min at 37°.

Discussion.—The striking similarity of the initiation of polyphenylalanine synthesis with N-acetylPhe-tRNA to that of viral protein synthesis with fMet-tRNA demonstrates that an efficient chain-initiating complex can be formed in the absence of fMet-tRNA and its codon. We suggested previously that acylation of the initiator aminoacyl-tRNA, a strong ribosome-messenger interaction, and a high magnesium concentration were conditions that induced formation of a chain-initiating complex. The suggestion appears to be still applicable here. The specific manner in which these conditions affect the initiation process, however, may not be as visualized originally, especially if the initiating complex with the 70S ribosome could be formed from dissociated subunits rather than directly from preformed 70S ribosomes.

Our observation that the factor sediments with the 30S ribosomal particles in a sucrose density gradient is somewhat similar to that made by Revel and Gros and Eisenstadt and Braverman on their initiation factors. The stimulation of N-acetylPhe-tRNA binding to ribosomes by the factor, and its fractionation pattern with DEAE-Sephadex, suggest that our factor preparation contains the factor designated as f2 by Salas et al. A GTP- and factor-dependent binding of the initiator aminoacyl-tRNA has been reported by a number of investigators.

The association of the factor with 30S rather than with 70S ribosomes may be regarded as compatible with the view that the 30S subunit initiates synthesis.

TABLE 2. Effect of factor on the binding of N-acetylPhe-tRNA.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Additions</th>
<th>Cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>Poly U</td>
<td>210</td>
</tr>
<tr>
<td>3</td>
<td>Poly U, factor, GTP</td>
<td>449</td>
</tr>
<tr>
<td>4</td>
<td>Poly U, factor</td>
<td>250</td>
</tr>
<tr>
<td>5</td>
<td>Poly U, GTP</td>
<td>196</td>
</tr>
</tbody>
</table>

The reaction mixture contained 0.05 M imidazole, pH 7.4, 0.004 M MgCl₂, 0.08 M NH₄Cl, 0.012 M 2-mercaptoethanol, 33 μg of low-factor ribosomes, 30 μg of N-acetyl-14C-Phe-tRNA and, where indicated, 25 μg of poly U, 0.001 M GTP, and 0.05 ml of factor, in a final volume of 0.25 ml. The reaction was stopped after 6 min at 37° by the addition of 3 ml of cold buffer solution containing 0.01 M imidazole, pH 7.4, 0.004 M MgCl₂, and 0.08 M NH₄Cl. The diluted mixture was immediately passed through a Millipore filter that was washed with 5 ml of the buffer solution, dried, and counted for radioactivity.
On the other hand, the association could have occurred in the preparation of the bacterial extract by a dilution of intracellular polyamines. Our study shows that the factor does not remain associated with the 30S ribosomes in the presence of spermidine at a concentration that is close to the intracellular concentration of the polyamine.23

Summary.—The initiation of polyphenylalanine synthesis with N-acetylPhe-tRNA by 70S ribosomes of E. coli requires some factor(s) that is released from the 30S particles by spermidine or putrescine, or both. The concentration of spermidine required to release the factor is within range of the intracellular polyamine concentration. The factor preparation contains the initiation factor designated as f2; it stimulates the binding of N-acetylPhe-tRNA to ribosomes in the presence of GTP and fractionates similarly to f2 on DEAE-Sephadex.

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Abbreviations: tRNA, transfer RNA; DNase, deoxyribonuclease; TCA, trichloroacetic acid; U, uridine; GTP, guanosine 5'-triphosphate; DEAE, O-(diethylaminoethyl); Tris, tris (hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

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‡ Operated by the University of Chicago for the Atomic Energy Commission.

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18 The most rigorous evidence for cyclic dissociation of ribosomes in protein synthesis appears to be that presented by Guthrie and Nomura.17 An alternative interpretation of their data is possible, however; i.e., 70S ribosomes that can equilibrate with free subunits preferentially bind (Met-tRNA, and those which cannot, bind Val-tRNA.
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