The Initial Phase in the Polyuridylic Acid-directed Polymerization of Phenylalanine

(Received for publication, February 27, 1967)

TOKUMASA NAKAMOTO

From the Department of Biochemistry, University of Chicago, Chicago, Illinois 60637

SUMMARY

The ribosome-polyuridylic acid-phenylalanyl-soluble ribonucleic acid complex has been shown to be an intermediate in the synthesis of polyphenylalanine. About half of the phenylalanine incorporated into polypeptide from the complex was located in the N-terminus. The formation of this ribosomal complex with phenylalanyl-soluble ribonucleic acid is rate-limiting in the synthesis of polyphenylalanine.

The polyuridylic acid-directed synthesis of polyphenylalanine from phenylalanyl-soluble ribonucleic acid has been shown to be stimulated upon preincubation of a cell-free system of Escherichia coli without guanosine triphosphate or enzymes (1-4). During the preincubation, phenylalanyl-sRNA becomes bound to the ribosomes (2, 5, 6). Presumably these two observations are directly related, the ribosome-poly U-phenylalanyl-sRNA complex being an intermediate in polyphenylalanine synthesis formed in a rate-limiting step. If this were the case, the isolated complex should exhibit enhanced activity. Moreover, if the synthesis of the polypeptide is started sequentially from the N-terminus as in protein synthesis (7), one would predict that the amino acid bound to the ribosome would be incorporated rapidly into the N-terminus of polyphenylalanine during subsequent polymerization.

We have carried out experiments to test these predictions, and have obtained results which show that the ribosome-poly U-phenylalanyl-sRNA complex is an intermediate in the synthesis of polyphenylalanine. A preliminary report of this work has been presented (8). Since then, Arlinghaus et al. have reported the synthesis of di- and tripeptides of phenylalanine in the rabbit reticulocyte system following stepwise binding of phenylalanyl-sRNA to the ribosomes (9).

EXPERIMENTAL PROCEDURE

E. coli B, grown in minimal medium, were harvested during midlogarithmic phase of growth as previously described (3). 1

Poly U was obtained from Miles Chemical Company, guanosine and adenosine triphosphates from P-L Biochemicals, and uniformly labeled L-phenylalanine from New England Nuclear. Phenyl isothiocyanate, trifluoroacetic acid, and N-ethylmorpholine were products of Eastman Organic Chemicals.

Preparation of Ribosomes—Ribosomes were isolated and first washed four times according to the procedure described by Allende, Monroe, and Lipmann (3). They were then washed two additional times, once in the extracting buffer containing 0.5% deoxycholate and finally in the extracting buffer.

Preparation of Phenylalanyl-sRNA—sRNA was isolated from E. coli and charged with phenylalanine by procedures previously described (3).

Preparation of Polymerizing Enzymes—The supernatant obtained after the separation of the ribosomes from the E. coli extract was treated with protamine sulfate, the maximal amount being added without precipitation of the transfer enzymes, and then fractionated with ammonium sulfate. The fraction precipitating between 50 to 63% saturation of ammonium sulfate was dissolved in 0.05 M Tris-HCl, pH 7.4, at a protein concentration of about 25 mg per ml and stored at -20°.

Isolation of Ribosome-Poly U-Phenylalanyl-sRNA Complex—The reaction mixture, containing about 1.5 mg per ml of ribosomes, 70 µg per ml of 14C-phenylalanyl-sRNA with a specific activity of 297 cpm per µM, 150 µg per ml of poly U, 0.05 M Tris-HCl, pH 7.4, 0.01 M MgCl2, 0.012 M mercaptoethanol, and 0.16 M NH4Cl, was incubated for 7 min at 25° and then cooled to 0°. A 1-ml aliquot was carefully layered over a 3.0-ml solution of 0.01 M Tris-HCl (pH 7.4)-0.01 M MgCl2-0.01 M β-mercaptoethanol, and 0.16 M NH4Cl. The mixture was then incubated at 37,000 rpm in a Spinco SW-39 rotor for 1 hour. After centrifugation, the tube was sliced about 8 mm from the bottom, and the complex concentrated in this lower portion of the tube was collected.

End Group Analysis of Polyphenylalanine by Modified Edman Degradation (10)—The polyphenylalanine for end group analysis was prepared by incubation of the isolated ribosome-poly U-14C phenylalanyl-sRNA complex with unlabeled phenylalanyl-sRNA, guanosine triphosphate, and enzymes. A typical reaction mixture contained 0.5 to 1.0 mg of labeled ribosomal complex per ml with 3000 to 4000 cpm of bound 14C-phenylalanyl-sRNA, 0.30 mg of unlabeled phenylalanyl-sRNA per ml, 0.05 M Tris-HCl (pH 7.4), 0.01 M MgCl2, 0.012 M β-mercapto-
ethanol, 0.4 mM guanosine triphosphate, 0.10 mM NH₄Cl, and about 100 µg per ml of the ammonium sulfate fraction of the supernatant. After incubation for 1 to 14 min at 25° during which time about 60% of the bound amino acid was polymerized, the reaction mixture was cooled to 0° and the ribosomes were isolated by centrifugation in a Spinco SW-39 rotor at 39,000 rpm for 2 hours. The ribosomes were then resuspended and heated for 10 min at 90° in 3% trichloroacetic acid. The resulting precipitate was collected by centrifugation, washed three times in 5% trichloroacetic acid, and once in ethanol-ether (2:1), and dried. Uniformly labeled polyphenylalanine was prepared in a similar manner, with the addition of 4°C-phenylalanyl-sRNA instead of unlabeled phenylalanyl-sRNA. The polypeptide was dissolved in a 1.0-ml solution of 4% sodium lauryl sulfate and of 4% N-ethylmorpholine, pH 9.0, and then 1 ml of pyridine was added to the suspension. After removal of undissolved particles by centrifugation, 0.03 ml of phenyl isothiocyanate was added to the solution containing the polypeptide. Following incubation at 40° for 2 hours, the solution was brought to dryness under reduced pressure and the dried residue was extracted four times with 1-ml portions of heptane. The residue was dried again under reduced pressure, a few drops of acetone being added to facilitate drying. The residue was dissolved in 1 ml of trifluoroacetic acid and kept at approximately 25° for 2 hours. The trifluoroacetic acid solution was cooled to 0° and then removed under reduced pressure. About 0.5 ml of heptane was added to the residue and the drying was continued under reduced pressure. After the residue was dried a second time following the addition of 0.5 ml of heptane, the phenyl isothiocyanat derivative of phenylalanine was extracted from the dried residue with heptane by extracting four times with 1-ml portions of the solvent. The combined heptane extract was dried in a planchet and its radioactivity was determined. The residue was resuspended in 0.5 ml of 4% N-ethylmorpholine, pH 10, and 0.5 ml of pyridine, and a 0.1-ml aliquot was dried in a planchet for radioactivity measurement.

End Group Analysis by Deamination (11)—To the polyphenylalanine sample dissolved in 0.05 ml of formic acid was added 0.05 ml of 10% acetic acid and 0.02 ml of 6 M NaNO₂. The polypeptide solution was kept at approximately 25° and 0.01 ml of NaNO₂ was added five times at intervals of 10 min. The deaminitized polyphenylalanine was then precipitated with 5% trichloroacetic acid and washed three times with ethanol-ether (2:1). The polypeptide was then dissolved in 0.05 ml of formic acid and 0.5 ml of concentrated HCl and hydrolyzed for 48 hours at 110° in a sealed tube. The hydrolysate was dried under reduced pressure and then chromatographed on Whatman No. 3 MM paper in 1-butanol-acetic acid-water (78:5:17). For radioactivity measurements, the chromatogram was cut into strips of 1-cm width and counted in a liquid scintillation counter.

RESULTS

In order to relate the formation of the ribosome-poly U-phenylalanyl-sRNA complex to the stimulation of polyphenylalanine synthesis observed on preincubation of the E. coli system, the complex was isolated by centrifugation, and its activity for polypeptide synthesis was measured. The results are presented in Fig. 1. It is clear that the isolated complex containing phenylalanyl-sRNA is more active in the synthesis of polyphenylalanine than ribosomes treated identically, except for the omission of phenylalanyl-sRNA during preincubation. More poly U was added to both systems after preincubation and isolation of the ribosomes to minimize any effect due to differences in the amount of poly U which remained bound to the ribosomes. Although only 68 cpm of radioactivity were bound to the complex added to each reaction mixture, the stimulation of polyphenylalanine synthesis at 2 min exceeded 600 cpm.

When the ribosomal complex formed with 14C-phenylalanyl-sRNA was isolated and incubated in the presence of 12C-phenylalanyl-sRNA and all the components required for peptide synthesis, about 60 to 90% of the labeled phenylalanine was rapidly incorporated into polypeptide. The kinetics of incorporation of the ribosome-bound phenylalanine is shown in Fig. 2. The incorporation was almost complete after the first minute. In this particular experiment 66% of the amino acid bound to the complex was incorporated into polyphenylalanine.

To determine if any of the ribosome-bound phenylalanine was polymerized, the isolated complex was heated for 15 min at 90° at neutral pH and the soluble product was chromatographed on Whatman No. 3 MM paper in pyridine-1-butanol-H₂O (2:2:1). The recovery of about 90% of the labeled amino acid as phenylalanine indicated that no significant polymerization occurred in the formation of the complex. Another property of the complex which was examined was the exchangeability of the ribosome-bound 14C-phenylalanyl-sRNA with phenylalanyl-sRNA free in solution. The complex was formed by incubating ribosomes with poly U and 14C-phenylalanyl-
sRNA and then 14C-phenylalanyl-sRNA was added to one reaction mixture and uncharged sRNA to another. Even upon further incubation, no significant decrease in radioactivity bound to the ribosomes was observed when the ribosomes were separated from free phenylalanyl-sRNA by sucrose density gradient centrifugation. The results are presented in Fig. 3. Likewise, no significant exchange of the ribosome-bound phenylalanyl-sRNA was observed when the labeled complex was incubated with 14C-phenylalanyl-sRNA at 25°C for as long as 10 min before the addition of the other components necessary for polymerization. Despite the large excess of 14C-phenylalanyl-sRNA, the amount of radioactivity incorporated into polypeptide by the system in 45 sec at 25°C was not significantly affected.

The incorporation of the phenylalanine bound to the ribosomes was followed by performing end group analysis on the polypeptide formed from unlabeled, free phenylalanyl-sRNA and the ribosomal complex containing bound 14C-phenylalanyl-sRNA. As a control, end group analysis was carried out on uniformly labeled polyphenylalanine prepared by preincubating and incubating the ribosomes with 14C-phenylalanyl-sRNA. The technique used for end group analysis was the Edman degradation, modified to analyze the highly insoluble polyphenylalanine. Even though sodium lauryl sulfate was used to solubilize the polypeptide, an effort was made to limit the chain length of the polyphenylalanine by short incubations. About 90% of the labeled polypeptide was solubilized by the detergent solution. The results of the end group analysis are presented in Table I. A preferential incorporation of the 14C-phenylalanine initially bound to the ribosomes into the N-terminus of the polypeptide is evident. Approximately 50% of the radioactivity incorporated from the isolated ribosomal complex was located in the amino end of the polyphenylalanine. In contrast, about 10 to 15% of the radioactivity of uniformly labeled polyphenylalanine was located in the N-terminus. The latter values indicate chain lengths of 7 to 10 residues for the polypeptide. This estimate of the chain length, however, is probably minimal since, with low radioactivity in the N-terminus, a small contamination in the extracted phenyl isothio-
Fig. 4. End group analysis by deamination. Preparation of the polyphenylalanine and analysis for N-terminal residue was carried out as described in “Experimental Procedure.” The profile on the left represents distribution of radioactivity in the hydrolysate of uniformly labeled polyphenylalanine, and the one on the right, that of the polypeptide prepared with labeled ribosomal complex and free, unlabeled phenylalanyl sRNA.

End group analysis was also carried out by deamination of the free amino group of the polyphenylalanine. Subsequent hydrolysis of the polypeptide released the N-terminal residue as β-phenyllactic acid. The radioactivity profile obtained from a chromatogram of the hydrolysate is shown in Fig. 4. In reasonable agreement with the results obtained by Edman degradation, only about 8% of the radioactivity in the uniformly labeled polyphenylalanine was located in the N-terminal, whereas approximately 45% of the radioactivity incorporated from the labeled complex was located in the N-terminal of the polypeptide.

DISCUSSION

The rapid and preferential incorporation of the ribosome-bound amino acid into the N-terminus of polyphenylalanine shows that the ribosome-poly U-phenylalanyl-sRNA complex is an intermediate in polyphenylalanine synthesis. Moreover, the enhanced activity of the isolated complex for polyphenylalanine synthesis indicates that the formation of this intermediate is rate-limiting.

The question of why only half of the phenylalanine incorporated from the complex is located in the N-terminus has not been resolved. Preliminary experiments in which the ribosomal complex was incubated with supernatant and guanosine triphosphate yielded only small amounts of di- and tripeptides with most of the amino acid being recovered as phenylalanine. Although this tends to rule out the alignment of two aminocyl-sRNAs next to each other in the isolated complex, more careful experiments will have to be carried out to rule out also the binding of a charged and stripped sRNA next to each other since the aminocyl-sRNA preparation contained significant amounts of the stripped sRNA. Another possible explanation for the incorporation of only half of the phenylalanine from the complex into the N-terminus is that only one of the two sites was occupied by an aminocyl-sRNA, and synthesis was initiated when the second aminocyl-sRNA either entered directly into the unoccupied site or entered the site which became available after the first aminocyl-sRNA was translocated, or both. These possibilities suggested that the other half of the phenylalanine incorporated from the complex might be located in the second position of the polypeptide. Unfortunately, our efforts to carry out a second Edman degradation on the polyphenylalanine were not successful.

Although it has recently been shown that protein synthesis in E. coli is initiated with N-formylmethionine (12, 15), a careful study of polyphenylalanine synthesis should be helpful in understanding the mechanism by which proteins are initiated. Synthesis of polyphenylalanine in cell-free extracts of E. coli occurs quite vigorously at a magnesium ion concentration optimal for the synthesis of viral protein (16). Presumably, under these conditions, synthesis of polyphenylalanine is initiated with phenylalanine, whereas synthesis of the viral proteins is begun only with N-formylmethionine. To account for the difference between the two systems, we have suggested that strong ribosome-poly U interaction enabled phenylalanine to initiate synthesis of polyphenylalanine by allowing the alignment of two phenylalanyl-sRNAs on the ribosome (17).

We are currently still trying to determine whether the ribosomal complex contains two sRNAs aligned next to each other. We are also studying the effect of adding N-acetylphenylalanyl-sRNA to the system synthesizing polyphenylalanine.

Acknowledgment—The author gratefully acknowledges the counsel of Dr. Fritz Lipmann of The Rockefeller University in whose laboratory this work was carried out.

REFERENCES

The Initial Phase in the Polyuridylic Acid-directed Polymerization of Phenylalanine
Tokumasa Nakamoto


Access the most updated version of this article at [http://www.jbc.org/content/242/19/4534](http://www.jbc.org/content/242/19/4534)

Alerts:
  - When this article is cited
  - When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/242/19/4534.full.html#ref-list-1](http://www.jbc.org/content/242/19/4534.full.html#ref-list-1)