A RIBOSOME-BOUND INTERMEDIATE IN POLYPEPTIDE SYNTHESIS*

Ralph Arlinghaus+, Gabriel Favelukes+ and Richard Schweet+

Department of Biochemistry, University of
Kentucky Medical Center, Lexington, Kentucky

Received March 20, 1963

Various enzyme fractions have been reported to catalyze the transfer of amino acids from C^{14}-amino acyl-RNA to protein (Bishop and Schweet, 1961; Fessenden and and Moldave, 1961; Lamfrom and Squires, 1962; Nathans and Lipmann, 1961; Takanami and Okamoto, 1960; von der Decken and Hultin, 1960). It is not clear from these earlier studies whether or not several enzymatic steps are involved in the transfer reaction. Several groups (Zamecnik, 1960; Fessenden and Moldave, 1962; G. Favelukes, cited in Schweet and Bishop, 1962) have noted the occurrence of ribosome-bound, C^{14}-amino acyl-RNA compounds. However, it has not been possible to demonstrate that these were intermediates in polypeptide synthesis. In this report, the transfer of C^{14}-phenylalanine from C^{14}-phenylalanyl-RNA to polyphenylalanine is shown to involve 2 steps catalyzed by different enzyme fractions.

C^{14}-phenylalanine transfer into "stable", polypeptide linkage was almost completely dependent upon the addition of poly U and soluble enzymes (Table I, lines 1-3). These results were dependent on pre-treatment of the ribosomes by pre-incubation and shock (Arlinghaus and Schweet, 1962) which lowers the endogenous hemoglobin synthesis of reticulocyte ribosomes. When purified enzymes were used, the formation of polypeptide required the addition of 2 enzyme fractions, TF-1 and TF-2 (Table I, lines 4-6). It should be noted that washing with both KCl and deoxycholate is required to remove the last traces of TF-2 from ribosomes. Since only a

*These studies were supported by grant H-5293 from the National Institutes of Health and grant G-21026 from the National Science Foundation.

+Postdoctoral fellow (RA) and Career research awardee (RS) of the National Institutes of Health, U.S.P.H.S. and postdoctoral fellow (GF) of the Consejo Nacional of the Argentine government.

single amino acid is incorporated, this provides evidence that 2 enzymatic steps are involved in the formation of polyphenylalanine from C^{14}-phenylalanyl-RNA.

TABLE I

TRANSFER OF PHENYLALANINE FROM C^{14}-PHENYLALANYL-RNA TO RETICULOCYTE RIBOSOMES

Assay Conditions	Incorporation in Ribosomes	
	Stable	Labile
1. Complete transfer system, AS_{70} enzymes	3670	2049
2. Minus soluble enzymes	85	588
3. Minus poly U	165	253
4. Complete transfer system, TF-1 and TF-2 enzymes	2519	3477
5. Complete, with TF-1 enzyme only	366	3881
6. Complete, with TF-2 enzyme only	115	455
7. Complete, zero time	16	295
8. Complete, boiled TF-1	79	535
9. Complete, TF-1 only, but C^{14}-leucyl-RNA	271	119
10. Complete, TF-1 only, plus Puromycin	180	3750

The complete transfer assay contained: 15 μmoles GSH, 0.1 μmole GTP, 10 μmoles creatine phosphate, 30 μg creatine kinase, 10 μmoles $MgCl_2$, 100 μmoles KCl, 50 μmoles Tris buffer pH 7.5; 100 μg poly U, 0.1 μmole L-C^{12} phenylalanine, 1.0 mg C^{14}-phenylalanyl-RNA containing 11,500 c.p.m. (prepared from uniformly-labeled C^{14}-phenylalanine, specific activity 15 μC/umole), and 3 mg of ribosomes (1.5 mg ribosomal protein) in a final volume of 1.5 ml. The mixture was incubated 10 minutes at 37°, diluted to 11 ml with cold medium B and the ribosomes removed by centrifugation. The ribosomal pellet was homogenized and precipitated with 5% TCA containing 10^{-4}M C^{12}-phenylalanine. The precipitate was homogenized again in 5% TCA, centrifuged and dissolved in 1 N NaOH. After 2 minutes at room temperature, the solution was neutralized and protein precipitated and washed. The supernatant radioactivity resulting from the NaOH hydrolysis is termed "labile" and radioactivity in protein is termed "stable", e.g. acid-insoluble after NaOH hydrolysis. The 2 samples were counted in a Tri-Carb liquid scintillation counter at 80% efficiency.

The ribosomes used were shocked with KCl and pre-incubated (2) to destroy incorporation into hemoglobin and then washed in a high KCl medium followed by a third wash in a deoxycholate medium to remove traces of soluble enzymes adsorbed to the ribosomes. Transfer fraction 1 (TF-1) and transfer fraction 2 (TF-2) are enzyme fractions previously described (3) and approximately 300 μg of each were used. Puromycin concentration was 6×10^{-5}M.

Ribosome-bound, C^{14}-phenylalanine having properties similar to C^{14}-phenylalanyl-RNA (termed "labile") was found when either crude or purified transfer enzymes were used. The formation of labile, ribosome-bound, C^{14}-phenylalanine required the addition of soluble enzymes and poly U, and of greater interest, was catalyzed by the TF-1, but not by the TF-2 enzyme fraction (Table I, lines 5 and 6). Boiled TF-1 was ineffective, no reaction occurred at 4°, and in other studies the

formation of this material increased with time and enzyme concentration. The specificity is shown by the failure of C^{14}-leucyl-RNA to form labile counts under the same conditions. Puromycin did not inhibit the reaction.

To study whether the ribosome-bound, labile C^{14}-phenylalanine was an intermediate in polyphenylalanine synthesis, ribosomes containing C^{14}-phenylalanine in labile linkage were prepared using TF-1, poly U and other components of the transfer system. The ribosomes were isolated by centrifugation (Table II). When these ribosomes were incubated in the complete amino acid incorporating system containing C^{12}-phenylalanine and transfer RNA, a large percentage of the labile C^{14}-phenylalanine bound to the ribosome was incorporated into stable, polypeptide linkage (Table II, lines 1 and 2). Since the total ribosome-bound radioactivity changed only slightly, and large amounts of free C^{12}-phenylalanyl-RNA were produced under these conditions, it appears that the labile C^{14}-phenylalanine did not leave the ribosome during this second incubation. This provides direct evidence that the ribosome-bound, labile C^{14}-phenylalanine is an intermediate in polypeptide synthesis. Addition of poly U stimulated the incorporation only slightly, and it is probable that poly U from the first incubation remains bound to ribosomes isolated under these conditions. When purified enzymes and the conditions of the transfer system were used, only TF-2 was required for the formation of stable, polypeptide radioactivity (Table II, line 4). However, it is not yet certain whether TF-1 used in the first incubation is removed from the ribosome by the isolation procedure used. Addition of C^{12}-phenylalanyl-RNA had no effect on polypeptide formation. When GTP and the generating system were omitted, no polypeptide formation occurred (Table II, line 5). It seems likely that a major function of GTP is in the formation of the peptide bond from the labile, ribosome-bound intermediate, Presumably, a GTP is split for each peptide bond formed, although the stoichiometry has not yet been studied. Only a small stimulation of the formation of the labile intermediate by added energy was noted. This observation requires further study. Puromycin inhibited the formation of polypeptide and in addition, in contrast to the other incubations, caused a loss of total C^{14}-phenylalanine from the ribosome (Table II, line 6). The radioactive material lost from the ribosome was acid-soluble, but was not de-

carboxylated by chloramine-T, e.g. was not free amino acid (Nathans et al, 1962). These results pinpoint the site of Puromycin action and provide a simplified system for studies of the detailed mechanism.

TABLE II

FORMATION OF POLYPEPTIDES FROM LABILE RIBOSOMAL INTERMEDIATE

Assay Conditions	Incorporation in Ribosomes		
	Stable	Labile	Δ
1. Not incubated	350	2300	0
2. Complete system, AS_{70} enzyme, transfer RNA, C^{12}-phenylalanine	1446	770	1096
3. Above, plus poly U	1600	620	1250
4. Transfer system, with TF-2	1050	1320	700
5. Above, minus energy	430	1840	90
6. Transfer system, plus Puromycin	600	430	250

Ribosomes were labeled as described in Table I, line 5, diluted 7-fold with medium B (1) and re-isolated. Labeled ribosomes were incubated for 20 minutes in the complete system (1), or in the transfer system described in Table I, except that 0.50 μmoles of GTP was used and C^{14}-phenylalanyl-RNA and poly U were omitted. Each tube contained 2 mg. of ribosomes. Data are given as in Table I. Minus energy refers to omission of GTP, creatine phosphate, and creatine kinase.

The requirement for 2 enzyme fractions for polyphenylalanine synthesis provides clear evidence for 2 chemical events in the transfer reaction. The evidence points to the labile, ribosome-bound C^{14}-phenylalanine as an intermediate in polyphenylalanine synthesis. It should be noted that the "labile" intermediate is also formed from C^{14}-leucyl-RNA when ribosomes which can synthesize hemoglobin are used (see ref. 9). A major difficulty in understanding, is the lack of knowledge of the detailed chemical events catalyzed by the enzyme fractions. In particular, TF-1 may catalyze a reaction required for, but not directly involved in, the formation of the "labile" intermediate.

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