TWO DISTINCT TRANSFER ENZYMES FROM RABBIT RETICULOCYTES WITH RIBOSOME DEPENDENT GUANOSINE TRIPHOSPHATE PHOSPHOHYDROLASE ACTIVITY

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Evidence is presented that indicates two reactions that each lead to the ribosome dependent hydrolysis of GTP by two physically distinct transfer enzymes from rabbit reticulocytes. One is dependent on aminoacyl-tRNA and the binding enzyme. The other is associated with TF-II activity and does not require aminoacyl-tRNA. The two enzymes differ in other enzymatic and physical properties.

It is of crucial importance to an understanding of the mechanism of protein synthesis to recognize which transfer enzymes carry out the hydrolysis of GTP during the formation of peptide bonds. It was previously reported that GTPase activity of enzymes from rabbit reticulocytes could be separated into three distinct fractions (1). The active component of one of these fractions has been purified to a point of apparent physical and enzymatic homogeneity and is the binding enzyme (TF-I), previously reported to have GTPase activity in partially purified preparations (2,3). The active component of the other fraction has been purified to a high degree and appears coincident with an enzyme required for the synthesis of a peptide bond to form diphenylanine from enzymatically bound phenylalanyl-tRNA. This enzyme has been called TF-II or peptide synthetase (3). Fellicetti and Lipmann (4) demonstrated GTPase activity associated with two fractions from rabbit reticulocytes that are required for polyphenylalanine synthesis. Klink and coworkers have reported ribosome dependent GTPase activity of similar fractions from calf and rat liver (5). Ravel has reported both F-I and F-II fractions from E. coli exhibit GTPase activity (6).

Here we report that the GTPase activities of the binding enzyme and TF-II from rabbit reticulocytes are highly dependent upon ribosomes and that these GTPase activities are associated with distinct and different physical and enzymatic properties. Both of these GTPase activities are specific for GTP. The GTPase activity of the third fraction we observed from rabbit reticulocytes (1) is not dependent upon ribosomes and appears to be related to one or more nonspecific nucleotidetriphosphate phosphohydrolases.

Materials and Methods: GTP-γ^{32}P and ATP-γ^{32}P were obtained from International and Chemical Nuclear Corporation. Materials and procedures have been described for isolation of phenylalanyl-tRNA, crude ribosomes, and crude enzymes (7,8). The term, phenylalanyl-tRNA, is used below to indicate unfractionated tRNA acylated with phenylalanine. Detailed procedures for purification of the enzymes will be presented elsewhere.

Crude ribosomes as isolated are heavily contaminated with transfer factors and nonspecific nucleotidetriphosphate phosphohydrolases present in the reticulocyte cell lysate. To reduce these activities to acceptable levels, the following procedure was used. To 300 mg of crude ribosomes in 15 ml, 15 ml of a solution containing 30% glycerol; 0.10 M Tris-HCl, pH 7.5; 0.02 M $MgCl_2$; and 0.06 M mercaptoethanol was added. Six ml of a 5% solution (W/V) of sodium deoxycholate (Mann Research Laboratories) was added and the mixture incubated for 10 minutes at 37°. This mixture was then brought to 200 ml final volume by addition of a solution containing 15% glycerol; 0.05 M Tris-HCl, pH 7.5; 0.01 M $MgCl_2$; and 0.03 M mercaptoethanol. Ribosomes were precipitated by centrifugation (50,000 RPM for 120 minutes) and resuspended in a small volume of 15% glycerol; 0.05 M Tris-HCl, pH 7.5; 0.01 M $MgCl_2$; 0.50 M KCl; and 0.03 M mercaptoethanol. After clarification at 25,000 x g for 10 minutes, the resulting clear supernatant was brought to 200 ml final volume of the same buffer. After gentle stirring, the ribosomes were precipitated by centrifugation, resuspended in 0.05 M Tris-HCl, pH 7.5; 0.005 M $MgCl_2$; and 0.001 M mercaptoethanol. Contaminating transfer factors were below detectable levels in these preparations.

Hydrolysis of GTP was measured by a procedure similar to that described by Conway and Lipmann (9) used with minor modification as previously described (10). The reaction mixture was incubated for 10 minutes at 37° and contained the following components in 0.50 ml: 0.02 M Tris-HCl, pH 7.5; 0.008 M $MgCl_2$; 0.07 M KCl; 0.01 M mercaptoethanol; 0.250 mg purified ribosomes; 4.0 µg binding enzyme or 3.6 µg TF-II protein; 2 mµmoles GTP-γ^{32}P (10^8 cpm/µmole) and 40 µg of phenylalanyl-tRNA where indicated. For heat inactivation, the enzymes were heated in 0.25 ml (binding enzyme, 5 minutes at 50°; TF-II, 10 minutes at 55°) containing 0.02 M Tris-HCl, pH 7.5; 0.008 M $MgCl_2$; 0.07 M KCl; and 0.01 M mercaptoethanol. After heating, tubes were chilled and the volume increased to 0.5 ml by the addition of components to give the amounts listed above. Then GTP hydrolysis was determined following subsequent incubation for 10 minutes at 37°. All values presented for GTP hydrolysis represent the average of at least three separate determinations.

Results and Discussion: The requirements for GTPase activity of the binding enzyme and TF-II are presented in Table I. Ribosomes assayed alone or with phenylalanyl-tRNA have little capacity to hydrolyze GTP. Neither enzyme

Table I. Requirements for GTPase activity of binding enzyme and TF-II.

Components	GTP Hydrolyzed µµmoles P_i Binding Enzyme	TF-II
Ribosomes only	1.1 ± 3.4*	1
Enzyme only	0	0
Ribosomes and phenylalanyl-tRNA	2	1
Enzyme and ribosomes	1	135
Enzyme and phenylalanyl-tRNA	2	0
Enzyme, ribosomes, and phenylalanyl-tRNA	91	130
Same, ATP for GTP	1	1

* Mean and standard deviation for 11 consecutive determinations.

preparation has detectable GTPase activity without ribosomes. These results reflect the removal of contaminating nonspecific nucleotidetriphosphate phosphohydrolases activity from the preparations of ribosome and enzymes. GTPase activity of TF-II is activated by ribosomes. No significant increase in GTP hydrolysis is observed if phenylalanyl-tRNA is added to the reaction

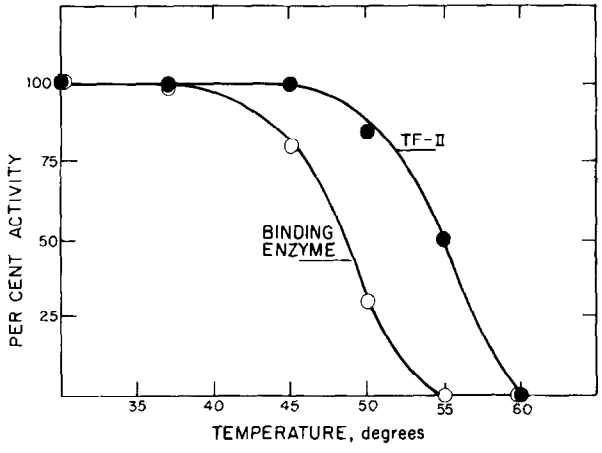

Figure 1. Heat inactivation of the two ribosome-dependent GTPases from rabbit reticulocytes. Enzymes were heated for inactivation as described in methods. Activity is expressed as a percent of the GTP hydrolyzed in tubes that were not heated. For the binding enzyme remaining GTPase activity was determined with 40 µg of phenylalanyl-tRNA.

mixture. This is in contrast to the GTPase activity of the binding enzyme which is dependent upon both ribosomes and phenylanyl-tRNA. Neither enzyme hydrolyzes ATP in place of GTP in the reaction mixture.

The binding enzyme and TF-II have different heat stability as indicated by Figure 1. For these experiments the enzymes were heated in solution containing the salts of the GTPase assay at the indicated temperature, then quickly chilled. The binding enzyme was heated for 5 minutes and TF-II for 10 minutes. The additional components of the GTPase assay reaction mixture were then added and GTPase activity measured as described in methods. Half of the GTPase activity of the binding enzyme is lost by heating at 47.5° for 5 minutes. Comparable loss for TF-II is at 55° for 10 minutes. Higher temperatures are required to inactivate the GTPase activity of TF-II if 5 minutes of heating are employed, however the effect of GTP in protection of the enzyme to heat inactivation, as demonstrated by the data presented in Table II, is less pronounced at higher temperature.

Both enzymes interact with GTP in the absence of ribosomes but in different ways. Data leading to this conclusion is presented in Table II. For these experiments binding enzyme or TF-II were heated at 50° or 55° respectively as described in methods except that GTP and phenylalanyl-tRNA were present during heating as indicated. GTP causes a marked decrease in the heat stability of the binding enzyme. Phenylalanyl-tRNA counteracts the effect of GTP so that when both GTP and phenylalanyl-tRNA are present increased heat stability of the binding enzyme is observed. These data are similar to the results reported by Ibuki and Moldave for aminoacyl transferase I activity from rat liver measured by the extension of nascent peptide chains (11). In contrast, the heat stability of the GTPase activity of TF-II is increased by GTP. Phenylalanyl-tRNA has little or no effect on the heat stability of TF-II. Studies to be reported in detail elsewhere indicate that GTP and phenylalanyl-tRNA effect the heat stability of the binding enzyme and TF-II for peptide synthesis in a manner similar to the effects shown here for the specific GTPase activity of these enzymes.

Also to be presented in detail elsewhere are data that indicate the molecular weight of the binding enzyme is 186,000$\pm$ 5,000 as determined by equilibrium centrifugation. The molecular weight of TF-II is about 70,000 as estimated by gel filtration. The corresponding sedimentation coefficients are 6.7 and 4.3 $S_{w,20}$ respectively. Both enzymes are required for the extension of globin peptides or the synthesis of polyphenylalanine.

We conclude that two distinct reticulocyte transfer enzymes have ribosome dependent GTPase activity. The enzymes appear to function in different reactions involved in the synthesis of peptide bonds. We remain reluctant to conclude that the formation of each peptide bond requires the

Table II. The effect of GTP and phenylalanyl-tRNA on the heat stability of the binding enzyme and TF-II.

Components present during heating	GTPase Activity After Heating μμmoles P_i	
	Binding Enzyme	TF-II
None	40	189
GTP	<5	398
Phenylalanyl-tRNA	42	196
GTP and phenylalanyl-tRNA	89	392
Enzyme not heated	103	390

The binding enzyme was heated at 50° for 5 minutes and TF-II heated at 55° for 10 minutes then assayed as described in methods except that GTP or 40 μg of phenylalanyl-tRNA were added as indicated to the 0.25 ml reaction mixture during heating. For the binding enzyme, 10 mμmoles GTP and for TF-II, 50 mμmoles GTP was added to the mixture. GTP-γ^{32}P was added after heating to give 1.5 (10^7) cpm/μmole. Components not added in the heating step were separately heated under the same conditions and added to the final 0.50 ml mixture. As determined in a separate experiment, less than 5 μμmoles or less than 15 μμ moles of GTP was hydrolyzed in the heating step with the binding enzyme or TF-II respectively. Remaining GTPase activity of the binding enzyme was determined with 40 μg phenylalanyl-tRNA in the final mixture. The final composition of the GTPase reaction mixture differs from that described in methods only in the amount of GTP present.

compulsory hydrolysis of more than one molecule of GTP; however, this possibility must be considered.

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