THE RELEASE OF PROTEIN FROM RETICULOCYTE RIBOSOMES

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The mechanism of polypeptide formation has been studied ir great detail in recent years through the use of cell-free systems. In general one can distinguish three stages of synthesis: 1) the activation of amino acids by ATP and amino acid specific enzymes, 2) the attachment of amino acids to soluble RNA through a high energy ester bond, and 3) the transfer of bound amino acids to the ribosome with concurrent formation of ribosomal bound polypeptide material.

The present investigation is concerned with yet another stage in protein synthesis, the release of completed protein molecules from the ribosome. This release, as studied in a cell-free system from rabbit reticulocytes, has been shown to be independent of the earlier steps in protein synthesis. The release specifically requires guanosine triphosphate (GTP) and possesses the character of an enzyme catalyzed reaction. It is independent of the processes of peptide synthesis.

Experimental: Ribosomes, soluble RNA and soluble enzymes were prepared from rabbit reticulocytes as described by Allen et al. (1962). The ribosomes were pre-incubated at 37° for 10 minutes in the presence of

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either C^{14}-valine or C^{12}-valine and the enzymes and other soluble factors of the complete system (Allen et al. 1962). The ribosomes were re-isolated and used in the two test systems, A and B, indicated below.

A C^{12}-valine ribosomes + C^{14}-valine → Incubated 20' x 37° as per Table I → Total C^{14}-protein

B C^{14}-valine ribosomes + C^{12}-valine → Incubated 20' x 37° as per Table I → Ribosomes / Soluble C^{14}-protein

Method A gave a measure of total amino acid incorporation into peptide linkage during the second incubation whereas Method B measured the release of polypeptide chains which were already present (either in complete or partially complete form) on the ribosomes before re-incubation. As will be seen from the results, the amino acid incorporation (A) may be stopped completely permitting an evaluation of the contribution of polypeptide synthesis to the release reaction as well as a measure of the release of finished protein from ribosomes which occurs independently of the formation of new peptide bonds. The incubation (20 min.) resulted in the release of 60 to 70 percent of bound radioactivity in test B (when all components of the complete system were present). Longer periods of incubation gave no further release. Incorporation in test A was also completed after 20 minutes of incubation.

Results and Discussion: The effects of changes in the composition of the incubation medium on the total incorporation (A) and release of soluble protein (B) are shown in table I. When both the ATP generating system and nucleoside triphosphates were omitted, neither process occurred. Both assay systems showed a strong dependence upon added GSH. Omission of soluble RNA or the amino acid mixture from the reactions results in

a reduction of the activity of each system to approximately one-half that of the complete system.

Significant differences between the results A and B were noted when soluble enzyme requirements and nucleoside triphosphate specificities were examined. Omission of soluble enzymes from assay A reduced the activity to 8 percent of the complete system. Test B, under these conditions, proceeded at 28 percent of maximal activity. The residual release activity (B) was dependent upon the presence of GSH. ATP, in the presence of soluble enzymes, had an almost equal effect in tests A and B. GTP, on the other hand, showed a selective effect in B which proved to be independent of factors other than those already bound to the ribosomes. Incorporation observed when GTP and soluble enzymes were added to test A was probably due to formation of ATP since addition of an ATP trapping system consisting of yeast hexokinase and glucose resulted in a further reduction of A without altering B.

The GTP-promoted release in test B does not depend upon any of the known soluble factors so far tested, such as soluble RNA, amino acid mixture or soluble enzymes which contain the activating enzymes and the transfer factor. The release observed in test B could not be attributed to the presence of contaminating C^{14}-valine-loaded soluble RNA since no net increase of radioactive protein was observed. Tests A and B show marked differences as to the inhibitory effects of trace amounts of bovine ribonuclease, release being relatively insensitive to the nuclease.

The specific effect of GTP upon test B may be seen in table 2. Under conditions where amino acid incorporation was reduced to an insignificant level, GTP was capable of promoting the release of protein. The other nucleoside triphosphates tested had only minimal activity. The rate of release was found to be half maximal with 12 mμ moles of GTP per ml. The reaction became saturated with respect to GTP

TABLE I. COMPARATIVE REQUIREMENTS OF AMINO ACID INCORPORATION AND PROTEIN RELEASE

No.	Nucleoside triphosphate content			Omissions from complete system	Amino acid incorporated	Protein† released
	ATP	GTP	Generating system			
	m molar	m molar			percent	percent
1.	1.0	0.25	+	None	100	100
2.	0	0	-	None	0	0
3.	1.0	0.25	+	Soluble enzymes	8	28
4.	1.0	0.25	+	GSH	8	10
5.	1.0	0	-	None	51	50
6.	0	1.0	-	None	5	26
7.	1.0	0	-	Soluble enzymes	0.7	13
8.	0	1.0	-	Soluble enzymes, soluble RNA and amino acid mixture	0.2	30

†Values of protein released have been corrected for labeled protein present in the supernatant fraction when nucleoside triphosphates and generating system were omitted (approx. 10%).

The complete system (No. 1) contained 50 **m molar** Tris Cl, pH 7.5, 50 m molar KCl, 4 m molar $MgCl_2$, 20 m molar GSH, pH 6.5, 5 m molar phosphoenolpyruvic acid, 20 µg. pyruvate kinase, 0.05 m molar each of 19 amino acids, 0.05 m molar C^{12}- or C^{14}-valine, saturating amounts of soluble enzymes (6 mg.) and soluble RNA (150 µg.), 3.0 mg. ribonucleoprotein and nucleoside triphosphates as indicated, in a volume of 1.0 ml.

with 100 mμ moles per ml. and was not changed by the addition of greater quantities. In the presence of excess amounts of GTP the reaction reached completion in 40 minutes at 37° with release of 26 percent of the ribosomal bound radioactivity.

While the enzymatic factor involved in the release of protein has not been isolated, the reaction characteristics suggest that an enzyme is required, i.e. the reaction has a definite time course, is dependent upon the presence of GSH, is unstable to prolonged storage at −18° and shows a specific requirement for GTP.

From the results reported here it is evident that two distinct steps may be observed in the release of protein from the ribosomal particle, each having different requirements. The first step requires those components of the cell-free system known to be involved in the incorporation of amino acids into polypeptide, such as the soluble enzymes, ATP, soluble RNA and the amino acid mixture. The effect of these components is demonstrated by the difference between No. 1 and No. 8 of table I and probably involves completion of primary polypeptide structure by the addition of the full complement of amino acids. The second step in the release process results in the appearance of soluble proteins under experimental conditions which do not permit polymerization of amino acids into polypeptide (No. 8 of Table I). For this second reaction those enzymes and soluble factors used in the first process are not essential but GTP is an absolute requirement.

The existence of preformed protein precursors bound to reticulocyte ribosomes has been reported by Rabinovitz et al. (1959). The release of protein, as discussed here, is thought to involve the participation of bound polypeptide in stages of protein assembly occurring subsequent to peptide bond formation.

The action of GTP reported here indicates that one locus of action of this nucleoside triphosphate is the release of protein from ribosomes.

TABLE 2. NUCLEOSIDE TRIPHOSPHATE SPECIFICITY OF PROTEIN RELEASE

Nucleoside triphosphate added	Amino acid incorporated	Protein released†
	c.p.m.	c.p.m.
GTP	5	237
ATP	2	26
CTP	3	49
UTP	1	49
None	0	0

Nucleoside triphosphate concentrations were 0.1 m molar. † Corrected as per table I.
Basic assay components were those of no. 8, table I (less GTP).
Labeled ribosomes contained 1785 c.p.m. C^{14}-protein.
Incorporation of C^{14}-valine in the complete system was 1605 c.p.m.

Release could require formation of secondary structure, or alternatively, the direct participation of GTP may be required in the reaction giving physical separation of the completed protein molecule from the ribosomal particle. While GTP may be required at more than one point in the synthetic pathway the previously supposed involvement of GTP in the transfer and polymerization reactions could be due to an absolute requirement for GTP in protein release. Failure of the release reaction might influence the rates of immediately preceding reactions so as to produce an apparent GTP requirement at several stages in the fully integrated test system normally used to measure protein biosynthesis.

Data, mentioned here in preliminary form, will be reported in detail elsewhere.

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