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Messenger RNA Dependent Synthesis of Peptides by Purified Bacterial Enzymes*

By
MIRKO BELJANSKI

Service de Biochimie Cellulaire, Institut Pasteur, Paris

With 3 Figures in the Text

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* Dedicated to Professor SEVERO OCHOA on the occasion of his 60th birthday.

Polypeptide synthetases purified from *Alcaligenes faecalis* catalyse an energy-dependent synthesis of peptides in the presence of an RNA fraction identified as messenger RNA. This RNA serves as an intermediate in peptide bond formation. It is capable of fixing directly the L-amino acids without competition and seems to act as template for determining the position of amino acids prior to their polymerisation into peptide chains. Heterologous RNA's and especially TYMV RNA show the ability to fix L-amino acids in the presence of purified enzymes from bacterial origin.

Introduction

On assaying in Dr. OCHOA's laboratory (1956—1958) enzyme fractions obtained in the course of purification of polynucleotide phosphorylase we found that certain fractions when combined with a particulate system from *Alcaligenes faecalis* stimulated the incorporation of amino acids into protein [1]. In fact two activities of these fractions (polynucleotide phosphorylase and amino acid incorporation) were found to increase together through several purification steps but were eventually separated from each other. The active fraction, originally named amino acid incorporation enzyme, was purified from *Alcaligenes faecalis* for reasons of convenience. In a cell-free system prepared from the same species this enzyme is involved in some phase of protein biosynthesis [1]. In the past few years we have described different properties of this enzyme [2,5]. One of its main characteristics is the capacity to synthesize various peptides from L-amino acids; a specific RNA and one of the four ribonucleoside-5'-triphosphates (ATP, GTP, CTP, UTP) must be present but neither bacterial membranes nor ribosomes are necessary. We named these enzymes polypeptide synthetases.

Abbreviations. m-RNA = messenger ribonucleic acid; RNA = ribonucleic acid; DNA = deoxyribonucleic acid; ATP, GTP, UTP, CTP = adenosine-, guanosine-, uridine-, cytidine-5'-triphosphates; TMV = tobacco mosaic virus; TYMV = turnip yellow mosaic virus; RN-ase = ribonuclease; P_i = orthophosphate; S-RNA= soluble ribonucleic acid; tris = tris(hydroxymethyl)aminomethane; DN-ase = deoxyribonuclease.

Methods

Preparation of polypeptide synthetases. The method for purification of polypeptide synthetases (amino acid incorporation enzyme) from *Alcaligenes faecalis* has been described [1]. Originally the enzyme assay was based on the stimulation of ^{14}C glycine incorporation by Alcaligenes particles. A new assay based on the exchange between ^{14}C labelled ribonucleoside-5′-diphosphates and homologous ribonucleoside-5′-triphosphates was elaborated [4]. In connection with the purification of the enzymes two important facts have to be stressed. First, the enzyme activity assayed with UTP, CTP, ATP and GTP can be found in both 40 and 70% ammonium sulfate fractions (step 5 [1]). The second fact is that the enzyme preparation has to be routinely treated with bentonite in order to eliminate the ubiquitous nucleases.

Table 1. *P_i release from ribonucleoside-5′-triphosphates*

	μmole P_i released	
	ATP	GTP
Complete system	0.40	0.21
Omit m-RNA	0.10	0.04
Omit L-ala	0.01	0.01
m-RNA after treatment with RNase	0.11	0.04
S-RNA instead of m-RNA	0.12	0.05
DNA instead of m-RNA	0.12	0.04

Reactants in μmoles per ml: ATP or GTP, 2.0; Ala, 6.0; Tris buffer pH 8.0, 100; $MgCl_2$, 5.0; KF, 2.0; m-RNA, S-RNA, DNA 30 μg; and enzyme, 300 μg. Incubation: 1 hr at 32°C.

Table 2. *Peptide formation*

	μmole ^{14}C-alanine peptide	
	in dipeptides	in tetrapeptides
Complete system	0.16	0.78
Omit m-RNA	0.12	0
S-RNA instead of m-RNA	0.12	0
DNA instead of m-RNA	0.16	0

Reactants in μmoles per ml: $MgCl_2$, 5.0; Tris buffer pH 8.0, 100; ATP, 2.5; L-alanine, 6.0 ($1.5 - 2.0 \times 10^5$ CPM); nucleic acid, 30 μg; enzyme, 300 μg. Incubation 2 hrs at 32°C.

Preparation of RNA. Total RNA containing pulse labelled RNA (*Alcaligenes faecalis* and yeast, labelled for 36 sec with ^{14}C-uracil) were isolated by the phenol method [4]. Alcaligenes faecalis RNA was fractionated with ammonium sulfate in order to concentrate messenger RNA [12]. Turnip yellow mosaic virus RNA was extracted by phenol from purified virus [14].

Liberation of P_i from ribonucleoside-5′-triphosphates. After incubation of the complete mixture (legend of the Table 1) the proteins were precipitated with trichloroacetic acid, eliminated by centrifugation and P_i in the supernatant was determined colorimetrically [4].

Peptides synthesis and analysis. The conditions for peptide synthesis are described in the legend of Table 2. The reaction was stopped with 1 ml of 10% of trichloroacetic acid. The proteins were removed by centrifugation and the supernatant

analysed for the presence of peptides. Separation of the peptides and of their products after acid hydrolysis has been achieved by paper chromatography using different solvents [4], followed by autoradiography. Average peptide length with ^{14}C alanine as substrate was determined using fluorodinitrobenzene [15].

^{14}C-amino acid-RNA complex. The conditions for the formation of the amino acid-RNA complex are described in the legend of the Table 3. The same conditions were used for the amino acid-RNA complex formation with each of the sucrose gradient fractions containing RNA. (Gradients centrifuged for 16 hrs in SW_{25} Spinco rotor.) The results are expressed as CPM, or millimicro-moles of ^{14}C-amino acid incorporated into RNA.

Polypeptide Synthetases

The polypeptide synthetases purified from *Alcaligenes faecalis* have the following properties:

a) Catalyse the L-amino acid dependent degradation of the four ribonucleoside-5'-triphosphates (ATP, UTP, CTP, GTP) into corresponding 5'-diphosphates and P_i (Table 1). Each ribonucleoside-triphosphate seems to be degraded by a separate enzyme.

b) Synthesize, in the absence of particulate material, α-linear peptides from free amino acids. Ribonucleoside-triphosphates and an RNA fraction which resembles or is identical with messenger RNA are required. This RNA accepts the L-amino acids and serves as a direct intermediate in peptide syntheses (Table 3).

Table 3. *^{14}C-amino acid-RNA complex (Alcaligenes faecalis)*

	mμmoles ^{14}C-aminoacid incorporated per mg RNA	
	Ala (ATP)	Leu (UTP)
Complete system	27.00	24.00
Omit enzyme	0.03	0.04
Omit energy (ATP or UTP)	0.05	0.04
Omit Mg^{++}	0.03	0.03
+ RNase	0.15	0.20
+ DNase	26.00	24.00
S-RNA instead of m-RNA	1.10	0.90
Poly AGUC instead of m-RNA	0.08	0.06

Reactants in μmoles per ml: $MgCl_2$, 4.0; Tris buffer pH 7.8, 50; ATP or UTP, 0.5; Purified Alcaligenes RNA (80% ammonium sulfate fraction), 20 μg; ^{14}C-amino-acid 1—2 mμmoles/ml = 60,000 CPM; pancreatic RNase, 1 μg; pancreatic DNase 20 μg. S-RNA or Poly AGUC (1 : 1 : 1 : 1), 200 μg. Incubated at 32° C for 20 min; complex washed with 0.3 N $HClO_4$ before counting.

c) Catalyse a Mg^{++}-dependent exchange of all four radioactive ribonucleoside-5'-diphosphates with homologous 5'-triphosphates, a reaction which appears to be related to their amino acid incorporating activity [3]. However, further investigation of this reaction is needed in order to correlate it with peptide bond formation.

Polypeptide synthetases seem to be widely distributed in nature. They have been purified from *Alcaligenes faecalis* [1], *Escherichia coli* [6] (Table 4), found in yeast [7] and detected in animal tissues [8,9]. Polypeptide synthetase appears to be different from the enzymes described by HOAGLAND et al. [16] which catalyse an amino acid dependent ATP-pyrophosphate exchange. Polypeptide synthetase does not show this reaction with 18 L-amino acids, except for occasional activity with valine and leucine. The exchange is obtained only with ATP, not with the other three ribonucleoside-5′-triphosphates.

Table 4. *^{14}C-aminoacid-RNA complex*

Energy source	mμmoles of ^{14}C-aminoacid incorporated per mg Alcaligenes RNA (60 % ammonium sulfate fraction)			
	E. coli enzyme			Alcaligenes enzyme
	Val	Phe	Leu	Leu
ATP	2.0	2.0	6.00	5.6
UTP	3.1	2.5	0.20	7.2
CTP	2.8	1.4	0.15	6.9
GTP	1.6	0.6	0.06	4.3

Reactants as in the legend of Table 3.

RNA requirement for polypeptide synthetase activity

In our early work the enzyme preparations contained 1—3 % of nucleic acids mainly ribonucleotides [2]. By further purification this nucleotide material could be nearly completely removed from the enzymes which in turn became inactive. Under proper conditions the amino acid dependent release of P_i from ribonucleoside-5-triphosphates and the peptide synthesizing activity could be restored by an RNA fraction. The peptides made from ^{14}C-alanine usually contain two and four residues of this amino acid (Table 2). These observations suggest that the active RNA might serve as intermediate in peptide synthesis. In fact, an amino acid RNA complex is easily isolated from the reaction mixture at acid pH. This test has been used to identify the active RNA. After sucrose gradient centrifugation of the total RNA containing pulse labelled (^{14}C-uracil) RNA, each fraction of the gradient was assayed for ability to fix ^{14}C-amino acids. Fig. 1 shows that ^{14}C-amino acid (AA-^{14}C-RNA complex) is bound to the rapidly labelled RNA. When pulse labelled total RNA is fractionated with ammonium sulfate only that fraction whose rapidly labelled component has base ratios identical to those of DNA from *A. faecalis* is active as acceptor RNA (Table 5 and Fig. 1).

Table 5. *Acceptor activity of alcaligenes RNA fractionated by ammonium sulfate* [12]

Fraction	Base ratio[1]	mμmoles of ^{14}C-aminoacid incorporated per mg RNA	
		Leu	Phe
40	1.04	1.2	1.0
60	1.03	4.0	3.18
65	2.18	24.0	12.5
90	2.15	26.0	14.2

[1] Measured by ^{32}P pulse labelling. Formation of aminoacid-RNA complex as indicated in the legend of Table 3.

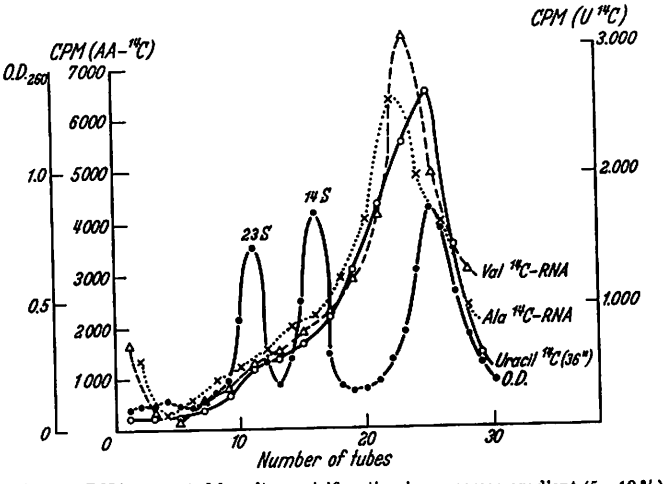

Fig. 1. Alcaligenes RNA separated by ultracentrifugation in a sucrose gradient (5—10 %). •——• optical density; o——o ^{14}C-uracil incorporated; ×·····× ^{14}C-valine-RNA complex; Δ------Δ ^{14}C-alanine-RNA complex. CPM of ^{14}C uracil are not substracted

Heterologous RNA as acceptor for amino acids

The ability of polypeptide synthetase to fix amino acids to RNA from *E. coli*, yeast and rat liver has been studied under the same conditions as were used with *A. faecalis* RNA. With each of these RNA's an amino acid RNA complex can be obtained. If the RNA's are separated on a sucrose density gradient, the rapidly labelled RNA is the fraction which is most active as amino acid acceptor. This is best illustrated by yeast RNA (Fig. 2). ^{14}C-amino acid is bound to the more rapidly sedimenting RNA fractions; none is in the region of transfer RNA (4S). RNA from TMV is practically inactive, while TYMV RNA has the capacity to fix amino acids in the presence of polypeptide synthetase. TYMV RNA isolated from purified virus shows a single peak on sucrose gradient analysis. The amino acids are fixed exclusively to the large RNA and not at all to the smaller RNA (Fig. 3). After degradation at pH 8.1 for 10 min

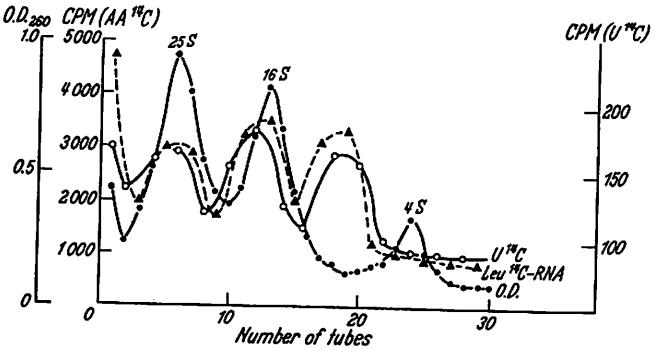

Fig. 2. Yeast RNA separated by ultracentrifugation in a sucrose gradient (5—10 %). •——• optical density; o——o ^{14}C-uracil incorporated; ▲- - - - - -▲ ^{14}C-leucine-RNA complex. CPM of ^{14}C-uracil are not substracted

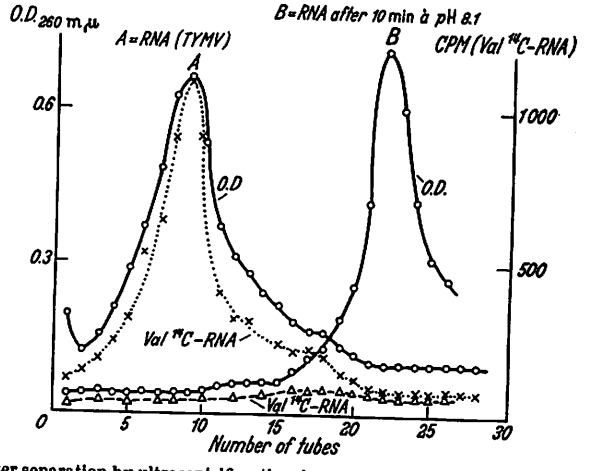

Fig. 3. TYMV after separation by ultracentrifugation in a sucrose gradient (5—10 %). A: o——o optical density of undegraded RNA; ×·····× ^{14}C-valine-RNA complex. B: o——o optical density of degraded RNA in tris buffer pH 8.1 for 10 min; △---------△ ^{14}C-valine-RNA complex. TYMV RNA is degraded when simply incubated in tris buffer as indicated [11]

TYMV RNA has a sedimentation constant close to 4 S and has lost all amino acid acceptor activity [11].

Linkage between amino acids and m-RNA

For amino acid RNA complex formation one of the four ribonucleoside-5′-triphosphates and polypeptide synthetase are required. The complex is stable to acid and is not dialysable. When incubated at alkaline pH (pH 9—11) ^{14}C-amino acid is liberated as free amino acid. These observations suggest that the amino acid is bound to m-RNA by an ester linkage. The ^{14}C-amino acid RNA complex was digested with spleen phosphodiesterase and RNA-ase, after which the products were

separated by electrophoresis and paper chromatography. For a given labelled amino acid all radioactivity was found to be associated with a single nucleotide. In the case of ^{14}C-valine fixed to RNA the isolated nucleotide carrying ^{14}C-valine has been found to be CMP [10]. Since the UV spectrum of the nucleotide-valine compound does not differ from that of the nucleotide alone and since the linkage is stable in acid, the linkage is probably between the carboxyl group of the amino acid and 2'OH of nucleotide ribose [10].

RNA as sequence template for amino acid incorporation

Polypeptide synthetase can fix all L-amino acids to messenger-RNA purified from *Alcaligenes faecalis*. Anyone of the four ribonucleoside-5'-triphosphates can serve as a source of energy, but there is some specificity for certain amino acids [4]. Fig. 1 shows that in the region in which rapidly labelled RNA is best separated from other types of RNA one finds approximately one alanine residue fixed per 140 nucleotides. The relative amounts of different amino acids fixed by a given quantity of RNA are very close to the relative amounts of the same amino acids in the total protein of this species [13]. A given L-amino acid is fixed independently of the presence or the absence of the other L-amino acids, i.e., without detectable competition. To inhibit by 20% the fixation of a labelled amino acid by a different ^{12}C-amino acid, a 3,000—5,000 times greater concentration of the latter is required. These results show that there are specific sites on the RNA for each amino acid, and that different enzymes, or at least different active sites, operate in fixing each amino acid. On the basis of these observations one can consider that active RNA can act as a template, since under optimal conditions one can fix as much as one amino acid per 6—10 nucleotides.

Concluding Comments

Polypeptide synthetases purified from *Alcaligenes faecalis* have the capacity of synthesizing peptides from free amino acids in the presence of "messenger-RNA" and ribonucleoside-5'-triphosphates. The L-amino acids are first fixed without competition to m-RNA by an ester linkage probably to the 2'OH of nucleotide ribose and an RNA amino acid complex is formed. This complex serves as intermediate in peptide bond formation. In the second step, for which ribonucleoside-5'-triphosphates are not required, peptide chains are formed from the amino acid RNA complex [10]. The amounts of amino acids fixed to m-RNA and the absence of competition between amino acids suggest that the messenger RNA may well be acting as a template for determining the position of amino acids prior to their polymerisation into peptide chains. Certain heterologous RNA are capable of accepting L-amino acids in the presence of enzymes

purified from *Alcaligenes faecalis*. The relationship between the present system and the *in vitro* system which requires ribosomes, supernatant and many other components is not clear at the present time.

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Dr. M. Beljanski,
Service de Biochimie Cellulaire, Institut Pasteur,
Paris XV, 25, Rue du Docteur Roux, France