Mechanisms of Extraribosomal Protein Biosynthesis in Eukaryotes

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The appearance of newly synthesized proteins in the total protein fraction of a cell-free protein-synthesizing system not containing ribosomes and mRNA and enriched with polyenzyme complexes of aminoacyl-tRNA synthetases and corresponding proteins is proved experimentally. For modeling of extraribosomal protein biosynthesis we proposed a molecular polyenzyme mechanism of protein biosynthesis on a protein matrix.

Key Words: *aminoacyl-tRNA synthetase; protein; biosynthesis; peptides; eukaryotes*

The molecular mechanism of protein biosynthesis on ribosomes of eukaryotic cells was extensively studied [10]. However, some authorities believe that under certain conditions simple low-molecular-weight proteins in human and animal cells can be synthesized on protein molecules as the matrix (*e. g.*, prions during prion diseases) [12,13].

Here we experimentally proved the possibility of extraribosomal protein biosynthesis in eukaryotes on protein molecules that serve as the matrix. The mechanism of this process was described.

MATERIALS AND METHODS

We used lysate of rabbit reticulocytes [1]. Large polyenzyme complexes from rat liver and brain were used to provide a variety of aminoacyl-tRNA synthetases (ARS), tRNA, and other proteins involved in protein synthesis [7]. Cell fractions (sedimentation coefficient 12S-30S) containing polyenzyme complexes with a molecular weight of 300-1000 kDa were mixed 1:1 with the reticulocyte lysate.

Polysomes and monosomes were removed from the mixture by centrifugation at 150*g* for 45 min. The supernatant with a sedimentation coefficient of 105S was collected, treated with 1 mM $CaCl_2$ and 50 U/ml micrococcal nuclease to remove endogenous rRNA and mRNA [10], transferred in 0.5-ml sterile plastic tubes, frozen in liquid nitrogen, and used as the initial material.

The initial material was defrosted before the experiment, and the main solution was prepared (Table 1).

The eukaryotic cell-free protein-synthesizing system (PSS) was prepared in sterile microcentrifuge tubes (Table 2).

Before incubation PSS (300 ml) was divided into 4 samples (75 ml). One sample was placed on ice and used as the control. Other samples were incubated in a thermostat at 30°C under sterile conditions. Aliquots of control and experimental samples (10 ml) were taken after 5, 15, and 30 h and placed on paper filters or used for inoculation in microbiological tests (5 ml). Filters were treated with 5% trichloroacetic acid, rinsed with distilled water, washed with alcohol, and dried. H_2O_2 (25 ml, 10% w/v) was placed on filters. Dry filters with samples were placed in scintillation vials with toluene scintillator, and radioactivity was measured. Vial radioactivity did not surpass 75 cpm (baseline).

RESULTS

Incorporation of radiolabeled amino acids into proteins was detected after 5-h incubation of PSS at 30°C

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TABLE 1. Composition of the Main Solution

Component	Initial concentration, mM	Volume, ml	Final concentration, mM
HEPES, KOH, pH 7.6	1000	200	200
Creatine phosphate	500	160	80
ATP	100	160	16
DTT	1000	10	10
NADH	50	50	2.5
D-1,6-fructose diphosphate	20	50	1
Mixture of individual amino acids (except phenylalanine)	—	300	0.25
Water	—	to 1000	—

for (Table 3). The degree of label incorporation is low and usually considered as the background. Researchers studying ribosomal protein synthesis in eukaryotic cells usually deal with protein fractions, whose radioactivity surpasses the this level by several orders of magnitude.

It should be emphasized that radioactivity of protein fractions before the experiment did not surpass the baseline level. Radioactivity of control samples remained unchanged throughout the experiment. However, after incubation of samples at 30°C radioactivity of protein fractions increased by one order of magnitude.

The absence of label incorporation into control samples indicated that labeled amino acids were not sorbed by protein fractions under these experimental conditions.

Protein synthesis was confirmed by positive dynamics of label incorporation into protein fractions of experimental samples throughout the experiment. Thus, proteins were synthesized under these experimental conditions and only protein molecules could serve as the matrix for assembly of amino acids into proteins.

For better understanding of this phenomenon we analyzed preribosomal stages of protein biosynthesis. ARS in mammalian cells are arranged in large polyenzyme complexes or aggregates. Some complexes were isolated and purified [2]. These complexes contain synthetases and other associated proteins.

We proposed the following 2-stage molecular mechanism that underlies protein synthesis on the protein matrix (PM) in PSS catalyzed by the dimeric enzyme ARS (Fig. 1).

In stage 1 only 50% substrate-binding sites in the enzyme molecule were active. Active centers in one of two subunits in free ARS (subunit 1) bind small substrates (ATP and amino acids) and the enzyme catalyzes the formation of aminoacyl adenylate. tRNA binds to subunit 1 and undergoes aminoacylation (Fig. 1, *a*). The molecule of aminoacyl-tRNA remains bound to the active center in subunit 1 until binding of tRNA to subunit 2 of the enzyme. Substrate-binding sites in subunit 2 of ARS bind small substrates, when aminoacyl-tRNA is bound to subunit 1.

Stage 2 in the absence of ribosomes and mRNA in PSS is characterized by deficiency of free amino acids (they are bound to tRNA), while the concentration of aminoacyl-tRNA in the reaction space increased. As a result, ARS (aminoacyl-tRNA still occupies active center of subunit 1) binds amino acid radicals in an open-chain protein molecule in the active center of subunit 2 (Figs. 1, *b*, *c*). This molecule now acts as PM.

Similarly, other ARS-aminoacyl-tRNA complexes bind other amino acid radicals in PM in active centers of subunits 2 and form a polyenzyme complex. In this complex consisting of PM molecule and corresponding ARS, active centers of ARS subunit 1 are associated with aminoacyl-tRNA, while active centers of subunit 2 are specifically bound to amino acid radicals in PM.

Since amino acid radical bound to the active center of subunit 2 in ARS belongs to PM, the carboxylic group of this amino acid forms the peptide bond. Therefore, the reaction of aminoacyl adenylate formation cannot proceed on subunit 2. The corresponding tRNA

TABLE 2. Cell-Free Protein-Synthesizing System

Component	Volume, ml	Final concentration, mM
Initial material	200	—
Main solution	30	—
NH_4Cl, 2 M	15	100 mM
$Mg(OA)_2$, 300 mM	10	10 mM
Phenylalanine (522 mCi/mmol, Amersham)	30	50 μ
Water	up to 300	—

TABLE 3. Kinetics of Incorporation of Labeled Amino Acid into PSS (cpm)

Series	Time, h		
	5	15	30
Control	95	107	84
I	548	704	1385
II	612	750	1239
III	390	486	897

does not bind to this subunit. These conditions promote binding of aminoacyl-tRNA to subunit 1 of ARS.

Under these conditions ester group of aminoacyl-tRNA-1 bound to one enzyme comes close to amino group of other amino acid residues (aminoacyl-tRNA-2) bound to the neighboring enzyme (Fig. 1, *b*). The amino group of amino acid residue of aminoacyl-tRNA-2 attacks the ester group in aminoacyl-tRNA-1 with the formation of a peptide bond between these two amino acid residues.

This mechanism of protein synthesis (similarly to ribosomal protein synthesis [2]) suggests that the formation of a peptide bond between amino acids does not involve special enzymes. The reaction depends on spatial proximity of 2'- or 3'-terminal regions of tRNA-1 and tRNA-2 and adjacent aminoacyl residues that act as reaction substrates.

In this reaction aminoacyl-tRNA-1 and aminoacyl-tRNA-2 serve as donor and acceptor substrates, respectively. Figure 2, *b* illustrates the formation of a peptide bond between amino acid residues of 2 aminoacyl-tRNA on adjacent (contacting) ARS.

Aminoacyl-tRNA is an activated macromolecular acyl derivative, which acts as a functional analogue of acyl enzyme groups. Therefore, the formation of a peptide bond results from proper spatial orientation and proximity of reacting groups in 2 aminoacyl-tRNA (similarly to protein synthesis on ribosomes). The conditions providing optimum orientation of interacting substrates can result from the thermal motion. The possibility for intradimeric (interdomain) mobility of ARS molecules cannot be excluded.

These data indicate that reaction requires optimum orientation of components and specific microenvironmental conditions. It cannot be excluded that X and Y groups localized close to aminoacyl-tRNA substrates properly oriented on ARS accept protons from NH groups of the acceptor thus increasing its nucleophilicity, and protonate carbonyl oxygen thus enhancing electrophilicity of the attacked donor carbons [14].

In living cells ribosomal protein synthesis is regulated by various mechanisms [15]. The regulation of total mRNA in cells is accompanied by drastic inhibition of ribosomal protein synthesis, while the formation of aminoacyl-tRNA remains unchanged [4]. This results in accumulation of aminoacyl-tRNA in cells and deficiency of free amino acids and tRNA.

Under these conditions peptide bond is formed between adjacent activated amino acids entering the composition of ARS-PM complexes and localized in close proximity to each other (Fig. 1, *c*). Protein biosynthesis proceeds in 2 stages (pathway II).

The mechanism of extraribosomal protein synthesis suggests that proteins are synthesized on the complex of ARS and PM via 3 (pathway I) or 2 (pathway II) consecutive chemical reactions.

In reaction 1 of pathway I, the amino acid carboxylic group interacts with ATP polyphosphate group with the formation of an aminoacyl adenylate anhydride bond (Fig. 2). In reaction 2, tRNA replaces adenylate residue with the formation of aminoacyl-tRNA ester bond. Reaction 3 yields amide (peptide) bond between the carboxylic group of aminoacyl residue in acyl-tRNA-1 and amino group of other aminoacyl residue in aminoacyl-tRNA-2.

Reaction 1 of pathway II yields aminoacyl adenylate (similarly to pathway I, Fig. 3, *b*). In reaction 2, amino group of aminoacyl adenylate-2 attacks the anhydride group of aminoacyl adenylate-1 with the formation of a peptide bond between aminoacyl residue-1 and aminoacyl adenylate-2 and release of AMP.

Studies of the energy balance showed that these reactions are not accompanied by free energy gain and therefore, the balance should not be shifted toward protein synthesis. However, this shift can be achieved at the expense of parallel pyrophosphate (PP) hydrolysis [5]. Under natural conditions PP is hydrolyzed by pyrophosphatase to orthophosphate (P):

$$n\text{PP}+n\text{H}_2\text{O}\rightarrow 2n\text{P}-n\times 30 \text{ kJ/mol}.$$

These data show that protein synthesis by pathways I and II is accompanied by a release of free energy. Therefore, this process is thermodynamically spontaneous and energetically supplied.

It should be emphasized that the total energy balance was calculated taking into account only the chemical mechanism of this process [6].

Energy dissipation into heat during protein synthesis on PM probably provides the proper spatial orientation, proximity of reacting groups, and optimum microenvironmental conditions. Experiments showed that the rate of extraribosomal protein synthesis is low. Therefore, complete synthesis of even small molecules takes much time.

Our experiments and published data [12,13] suggest that extraribosomal protein synthesis in eukaryo-

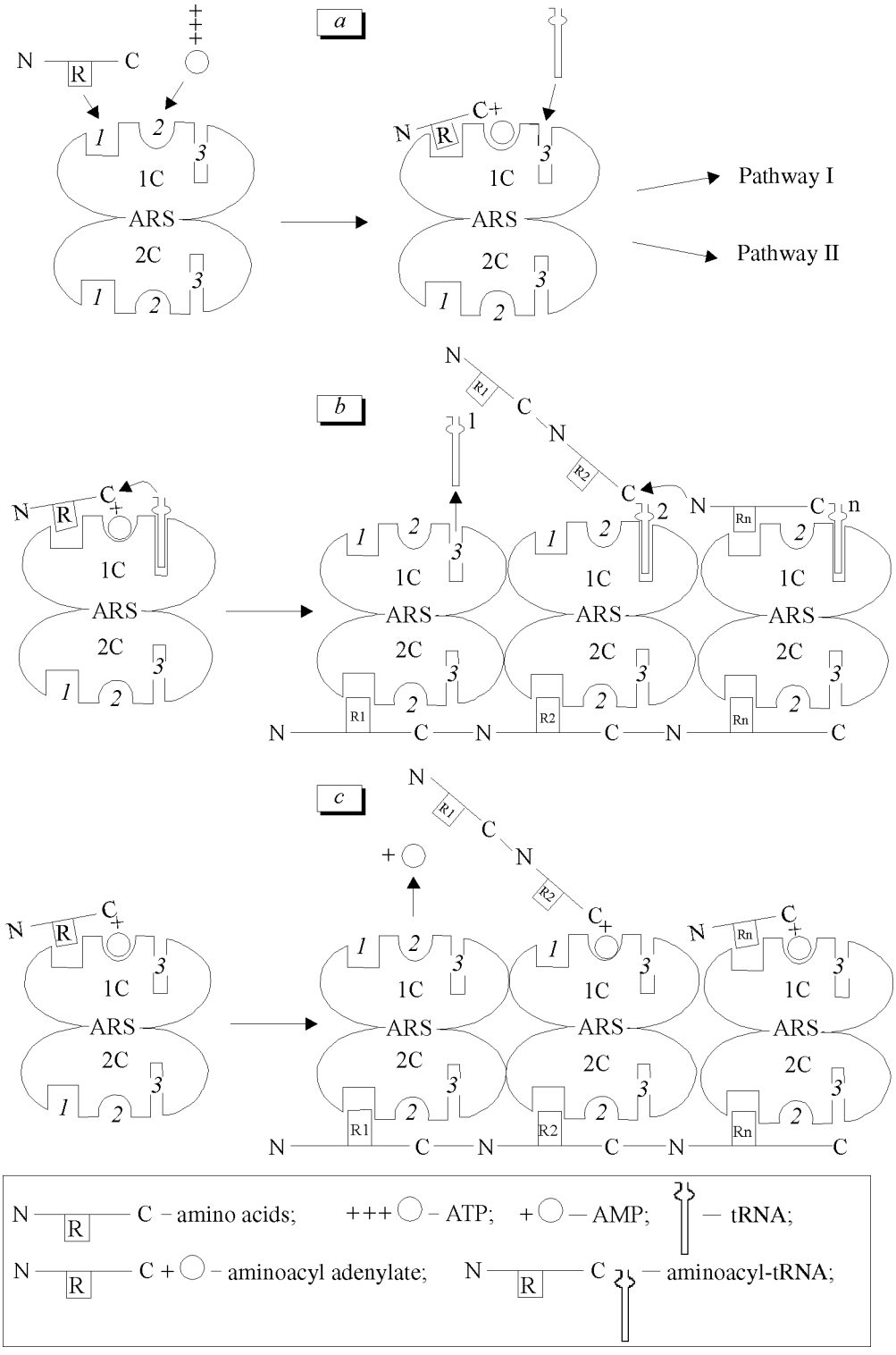

Fig. 1. Mechanism of protein biosynthesis on a protein matrix. *a*) formation of aminoacyl adenylate and aminoacyl-tRNA catalyzed by aminoacyl-tRNA synthetases (ARS) and followed by the formation of a peptide bond between activated amino acids. *b*) pathway I: formation of peptide bonds between activated amino acids in the ARS complex and protein matrix. *c*) pathway II: formation of peptide bonds between activated amino acids in the aminoacyl adenylate-ARS complex and protein matrix. Active centers for binding of amino acids (*1*), ATP (*2*), and tRNA (*3*). Complexes 1 (1C) and 2 (2C) in active substrate-binding sites of ARS.

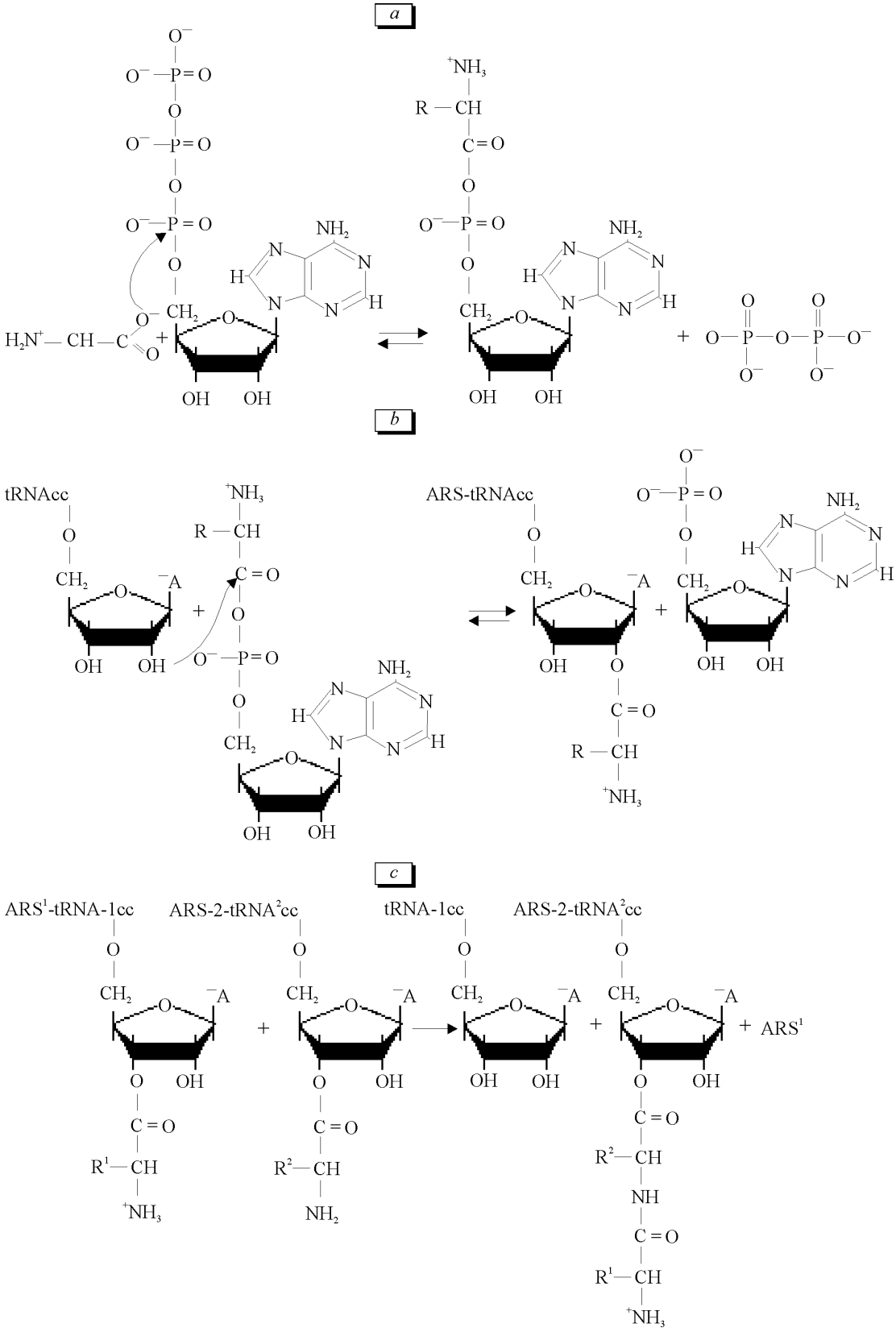

Fig. 2. Formation of peptide bond between activated amino acids with participation of aminoacyl-tRNA. *a*) carboxylic group in amino acids interacts with polyphosphate group in ATP with the formation of anhydride bond in aminoacyl adenylate. *b*) tRNA replaces adenylate residue with the formation of ester bond aminoacyl-tRNA. *c*) formation of amide (peptide) bond between carboxylic group in aminoacyl residue of aminoacyl-tRNA-1 and amino group in aminoacyl residues of aminoacyl-tRNA-2. Here and Fig. 3: ARS, aminoacyl-tRNA synthetase.

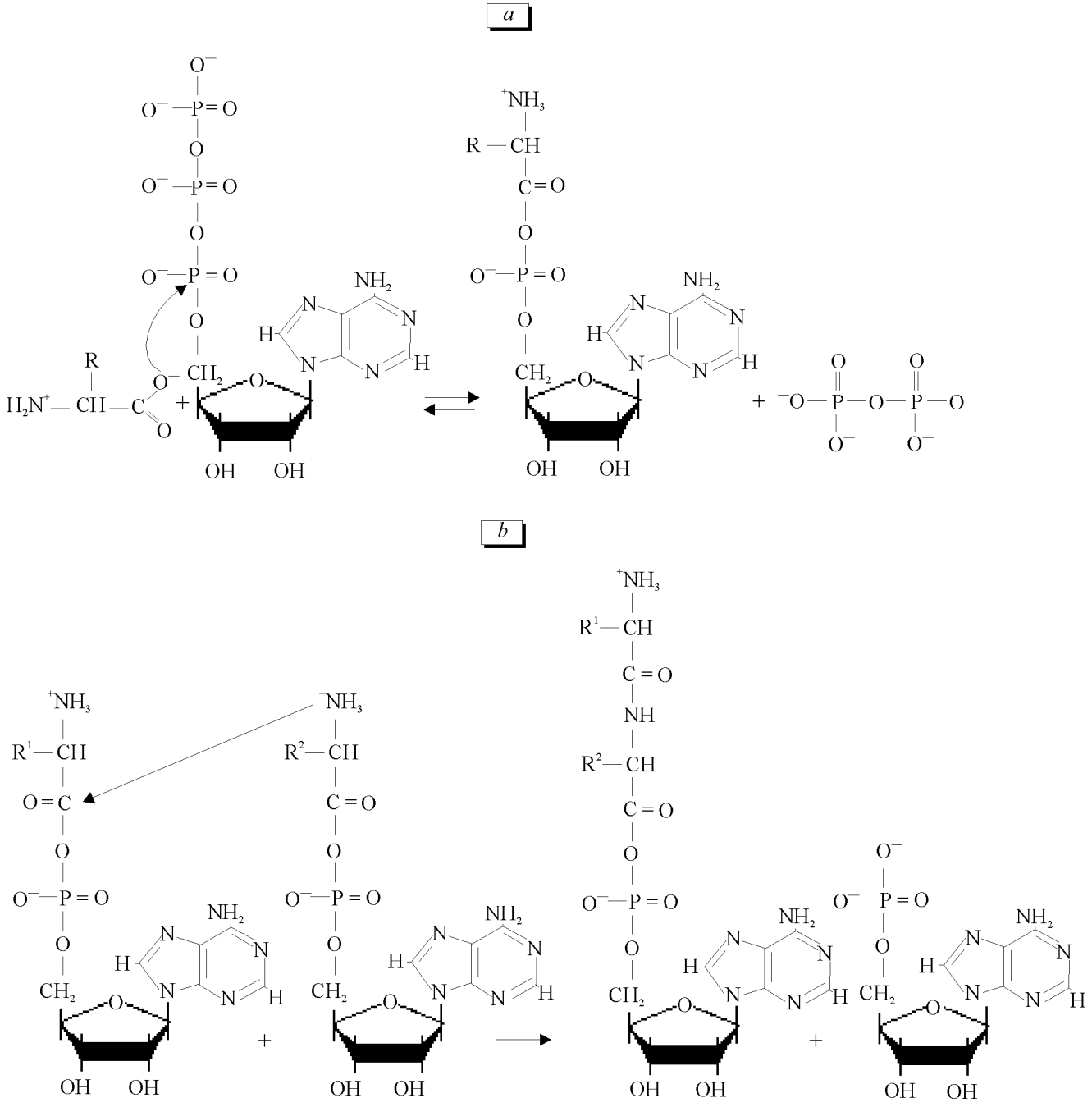

Fig. 3. Formation of peptide bond between activated amino acids without involvement of aminoacyl-tRNA. *a*) formation of aminoacyl adenylate. *b*) amino group in aminoacyl adenylate-2 attacks anhydride group in aminoacyl adenylate-1 with the formation of peptide bond between aminoacyl residue-1 and aminoacyl adenylate-2 accompanied by AMP release.

tes is a primitive process that evolutionarily precedes ribosomal protein synthesis.

In eukaryotic cells ribosomes ensure high rate and reliability of protein synthesis. Therefore, extraribosomal protein synthesis does not proceed in normal cells. However, extraribosomal protein synthesis (cell atavism) can be initiated under certain extreme conditions. This results in the formation of small and structurally simple proteins, which are accumulated near polyenzyme protein-synthesizing complexes in eukaryotic tissues. Microscopic examination showed that certain human and animal diseases are accompanied by the appearance of these agglomerates (patches) consisting of low-molecular-weight proteins [8,9].

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