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SYNTHESIS OF LENS PROTEIN *IN VITRO* VI. METHIONYL-tRNA FROM EYE LENS

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1. Introduction

Methionyl-tRNA_F has been isolated from bacteria [1] mitochondria [2] and chloroplasts [3]. This tRNA species initiates polypeptide chains in prokaryotic cells [4]. In eukaryotes evidence is accumulating that methionyl-tRNA is involved in the initiation of perhaps all cytoplasmic proteins [5, 6] in spite of suggestions that N-acetyl-valine [7], N-acetyl-glycine [8] and N-acetyl-serine [9] play the role of initiators of protein biosynthesis. A blocked cysteinyl-tRNA which accumulates on rabbit reticulocyte ribosomes after NaF treatment has also recently been reported [10]. In relatively short nascent peptide chains of e.g. hemoglobin methionine has been detected in the NH₂-terminal position [11]. However, in the mature protein methionine is removed enzymatically, presumably already in an early stage of peptide chain growth. It has been stressed earlier that the eye lens is a most useful tool for the study of cell differentiation at the molecular level, as the process of differentiation occurs during the whole life span of the lens [12]. We described the properties of the lens cell-free system previously [13-16].

Structural studies of the major lens protein, α -crystallin, revealed that acetylated methionine occurs in the NH₂-terminal position [17, 18]. In connection with this striking feature a search was made for initiator tRNA in lens tissue. This paper describes the isolation of three met-tRNA's from calf eye lens.

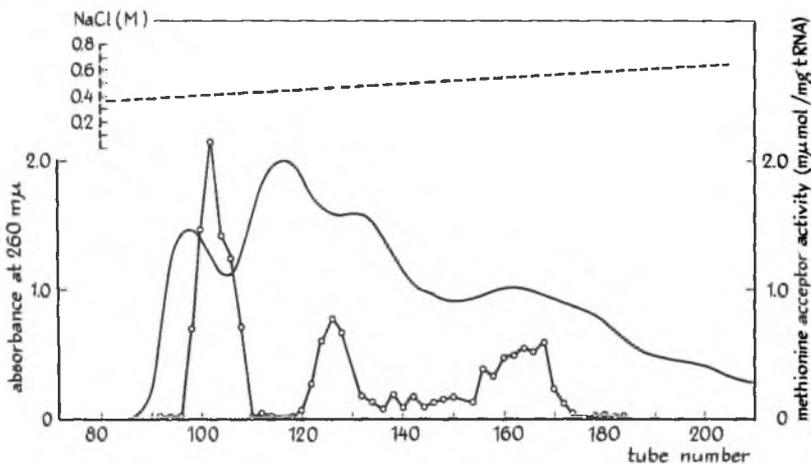
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Only one of these species can be charged by *E. coli* synthetase and formylated by *E. coli* formylase. Formylation of ³⁵S-met-tRNA_I results in an enhancement of radioactivity in newly synthesized peptide chains. The formylated met-tRNA_I initiates polypeptide formation in a crude lens cell-free system. The NH₂-terminal dipeptide contains the same amino acids as the N-terminus of α -crystallin. The initiator properties of met-tRNA_I is also demonstrated by binding experiments with AUG and its behaviour in the presence of antibiotics.

2. Materials and methods

2.1. Preparation of bovine eye lens tRNA

After isolation of the lenses, the capsules containing the epithelial cells and the outer cortexes were collected. Homogenization of the combined material was carried out in 0.008 M MgCl₂, 0.025 M KCl and 0.05 M KCl and 0.05 M Tris-HCl, pH 7.6. The homogenate was centrifuged at 12,000 g for 20 min. The supernatant was applied to a discontinuous sucrose gradient [19] and centrifuged at 78,000 g for 16 hr at 2°. The supernatant was shaken with an equal volume of 80% phenol and 1% SDS. This phenol extraction was repeated twice. The aqueous layers were combined and the RNA was precipitated with 2.5 volumes of ethanol. The tRNA was freed from traces of protein and phenol on a Sephadex A-50 column. The amino acid assay and formate acceptor activity measurement of the tRNA's was performed with crude synthetase from *E. coli*



1. Chromatography on benzoylated DEAE cellulose of crude eye lens tRNA. The RNA's were eluted with a linear gradient of ml 0.3 and 300 ml 0.7 M NaCl in the presence of 0.01 M magnesium-acetate and 0.005 M β -mercaptoethanol. 2.5 ml fractions were collected and tested for methionine acceptor activity. — : absorbancy at 260 nm; \circ - \circ - \circ : methionine acceptor activity assayed with crude rat liver tRNA synthetase. The three peaks of methionine acceptor activity are successively met-tRNA_I, met-tRNA_{II} and met-tRNA_{III}.

pared according to the procedure of Muench Berg [20] and crude synthetase from rat liver following the procedure described by Takeishi et al. [21]. 14 C-formate (specific activity 40 mCi/mole) was activated using a dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction of pigeon liver supernatant [4], and radioactive aminoacyl-tRNA's were prepared from the purified fractions (^{35}S , specific activity 10–14 Ci/mole).

Preparation of the cell-free system

Fresh calf bovine eyes were obtained from the slaughterhouse. The lenses were removed with capsules intact. The epithelial and outer cortex were collected in a medium containing 0.35 M sucrose, 0.05 M HEPES, pH 7.0, 0.005 M magnesium-acetate, 0.005 M β -mercaptoethanol and 0.150 M potassium acetate. After homogenization the lysate was centrifuged at 1000 g during 10 min and used in the incubation mixture.

Results and discussion

Figure 1 shows that chromatography of tRNA's from

bovine eye lens on benzoylated DEAE-cellulose results in a clear separation of three fractions, namely tRNA_I^{met}, tRNA_{II}^{met}, and tRNA_{III}^{met} which accept radioactive methionine. Each fraction can be acylated with the amino acid with the aid of rat liver synthetases. However, only the first fraction can be also esterified by *E. coli* methionyl-tRNA synthetase. The magnesium concentration dependence of the acylation reaction for the three species is shown in fig. 2.

The incorporation of methionine into hot TCA precipitable material is demonstrated in fig. 3. Incorporation by met-tRNA_I is low as compared to met-tRNA_{II} and met-tRNA_{III}. However, a five-fold increase can be achieved after formylation of met-tRNA_I. Actually this tRNA is the only species which can be formylated enzymatically.

In order to test the reliability of the incorporation experiments the effect of a number of antibiotics was studied. The results are summarized in table 1. It is obvious that chloramphenicol does not inhibit the incorporation of methionine by either of the tRNA species. From this observation the conclusion can be drawn that no mitochondrial peptide formation took place and that bacterial infection may be excluded. Aurintricarboxylic acid at a concentration of 2×10^{-4} M inhibits initiation

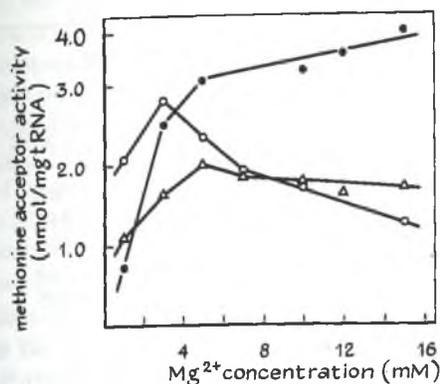


Fig. 2. Effect of Mg^{2+} concentration on the acylation reaction. The reaction mixture contained 0.6 μ moles ATP, 6 μ moles Tris-HCl, pH 7.4, 7.5 μ moles KCl, $1-2 \times 10^{-3}$ μ moles ^{14}C -methionine, 50–150 μ g crude aminoacyl tRNA synthetase from rat liver or *E. coli* and 10 μ g eye lens tRNA in a total volume of 150 μ l. The incubation was for 15 min at 37°. Symbols represent the following: $\circ-\circ-\circ$: methionine acceptor activity of $tRNA_I$ assayed with rat liver synthetase, $\bullet-\bullet-\bullet$: methionine acceptor activity of $tRNA_I$ assayed with *E. coli* synthetase, $\triangle-\triangle-\triangle$: methionine acceptor activity of $tRNA_{II}$ and $tRNA_{III}$ assayed with rat liver synthetase.

Table 1
Effect of antibiotics on protein synthesis.

Addition	Met-tRNA _I	Met-tRNA _{II}	Met-tRNA _{III}
None	100% (318 cpm)	100% (2145 cpm)	100% (1515 cpm)
A.T.A. 2×10^{-4} M	15%	60%	61%
cycloheximide 200 μ g/ml	2%	2%	3%
chloramphenicol 100 μ g/ml	105%	109%	95%

Reactions are described in the legend to fig. 3. Total volume of the incubation mixture was 30 μ l, the incubation time was 10 min at 37°.

preferentially [20]. It appears from table 1 that addition of this compound to the incubation mixture mainly interferes with incorporation by met-tRNA_I. On the other hand, the addition of cycloheximide results in an almost complete inhibition of incorporation independently of the type of met-tRNA used.

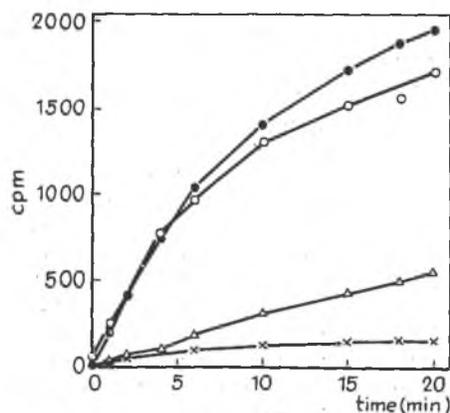


Fig. 3. Transfer of ^{35}S -methionine from met-tRNA species into protein. 1.4×10^5 cpm of eye lens ^{35}S met-tRNA were incubated at 37° in a final volume of 0.22 ml with 0.120 ml 15,000 g eye lens extract. All reactions further contained per ml 1.0 μ moles Mg-ATP, 0.5 μ moles GTP, 5.0 μ moles β -mercaptoethanol, 10 μ moles creatine phosphate, 1.0 mg creatine-phosphokinase, 25 μ moles HEPES (pH 7.0), 50 μ moles KCl, 2.0 μ moles magnesium-acetate and 0.1 μ moles of all twenty amino acids except methionine. 20 μ l samples were withdrawn at intervals and tested for alkali stable, acid precipitable radioactivity on glass fiber filters. $\times-\times-\times$ met-tRNA_I, $\triangle-\triangle-\triangle$ Fmet-tRNA_I, $\circ-\circ-\circ$ met-tRNA_{II}, $\bullet-\bullet-\bullet$ met-tRNA_{III}.

As this antibiotic inhibits initiation equally well as translation on 80 S ribosomes the effect observed is easily understood.

Studies with *E. coli* showed that the initiator met-tRNA_F binds better to ribosomes at lower Mg^{2+} concentration than met-tRNA_M [23–25]. Using *E. coli* ribosomes and the triplet nucleotide ApUpG we found similar properties for the eye lens tRNA's (table 2). It appears that the ability for binding of met-tRNA_I is not affected by blocking of the NH_2 -group of methionine.

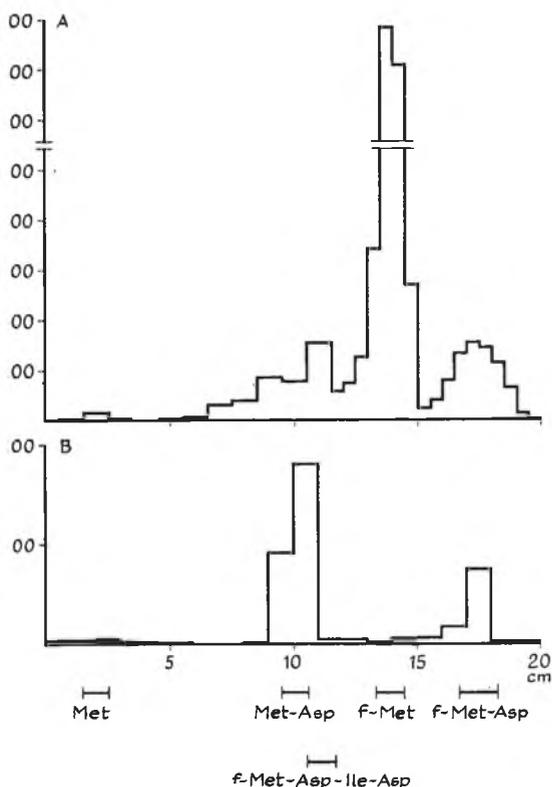
The results presented demonstrate that there occur three chromatographically distinct methionyl-tRNA's in the lens, one of which seems to function in protein chain initiation. Our structural studies revealed that both acidic and basic peptide chains of α -crystallin contain N-acetyl-met-asp in NH_2 -terminal position [27, 28].

When formylated met-tRNA_I is incubated with the crude cell-free system, as described under fig. 3, the sequence f-met-asp is found in the NH_2 -terminus

Table 2
Binding of eye lens methionyl-tRNA species to ribosomes in the presence of ApUpG.

[g^{2+} conc. nM)	Met-tRNA _I		F-Met-tRNA _I		Met-tRNA _{II}		Met-tRNA _{III}	
	(cpm)	(cpm)	(cpm)	(cpm)	(cpm)	(cpm)	(cpm)	(cpm)
	+ AUG	- AUG	+ AUG	- AUG	+ AUG	- AUG	+ AUG	- AUG
5	35	50	305	290	45	60	130	160
8	365	40	630	300	55	55	210	240
2	2100	45	2230	490	200	65	340	260
5	3315	215	4630	910	680	440	630	310
0	4010	685	4965	1600	1465	570	1200	365

binding reaction contained in a 40 μ l volume: 1.0 A_{260} units of washed *E. coli* ribosomes, 0.05 A_{260} units of ApUpG, 0.1 M HCl, pH 7.5, 0.05 M KCl, 0.005 M β -mercaptoethanol, 0.5 pmole 35 S-met-tRNA_I, 0.5 pmole Formyl- 35 S-met-tRNA_I, 0.5 pmole 35 S-met-tRNA_{II} and 0.5 pmole 35 S-met-tRNA_{III} (1 pmole = 20,000 cpm). The mixture was incubated for 8 min at 37° filtered on nitrocellulose membranes according to the procedure of Nirenberg and Leder [26].



of the newly formed polypeptide (fig. 4). Removal of the formyl group results in the appearance of a spot identified as met-aspartic acid (fig. 4B). As we failed to detect an acetylated met-tRNA species in lens one may still question which one of the three methionyl-tRNA's supplies the N-terminal methionine in native α -crystallin.

In most mammalian proteins the NH_2 -terminal methionine is removed shorter or later after initiation. If this situation would occur in α -crystallin synthesis, an NH_2 -terminal sequence met-met-aspartic acid has to be expected. In that case we should have found F-met-met-(asp) instead of F-met-aspartic acid. As the latter peptide is found as an NH_2 -terminal

Fig. 4. Pronase digestion of formyl- 35 S-methionyl product. After 20 min incubation at 37° (fig. 3) pancreatic ribonuclease (0.1 mg/ml) and EDTA (0.03 M) were added and incubation continued for 10 min. The sample was precipitated with trichloroacetic acid, centrifuged and the precipitate washed three times with 5% TCA and once with ethanol. The precipitate was resuspended in 1 ml 0.1 M NH_4HCO_3 and 2 mM $CaCl_2$ containing pronase at 0.2 mg/ml. After incubation at 37° for 6 hr a suspension of 1 ml Dowex 50 resin in H^+ form was added. The water layer containing the blocked peptides was lyophilized and analyzed by paper ionophoresis at pH 6.5 (A). The spot of radioactivity migrating together with cold formyl-methionyl-aspartic acid was cut out, eluted from the paper and treated with 0.5 M HCl for 20 min at 90° to remove the formyl residues. After freeze-drying the deformedylated product was again analyzed by ionophoresis at pH 6.5 (B).

peptide, one has to conclude that the methionine present has been donated by the initiator tRNA in native α -crystallin. This residue may remain in the NH_2 -terminal position due to the nature of the met-asp bond. In *E. coli* B [29] it has been demonstrated that the ribosome-bound aminopeptidase cleaves all methionine dipeptides tested with exception of met-asp.

An alternative explanation is the assumption that acetylation takes place before the methionine residue becomes accessible to the attack of aminopeptidase. In fig. 4A radioactivity which migrates slower than F-met is also found. The main activity coincides with the region of F-met-asp-ile-ala. As the NH_2 -terminal tetrapeptide of all α -crystallin subunits is acetyl-met-asp-ile-ala [27] this is an additional indication for faithful initiation.

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