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

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1. Introduction

Methionyl-tRNA\textsubscript{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 NH\textsubscript{2}-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, \(\alpha\)-crystallin, revealed that acetylated methionine occurs in the NH\textsubscript{2}-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\textsubscript{F} from calf eye lens.

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Only one of these species can be charged by \textit{E. coli} synthetase and formylated by \textit{E. coli} formylase. Formylation of \textsuperscript{35}S-met-tRNA\textsubscript{F} results in an enhancement of radioactivity in newly synthesized peptide chains. The formylated met-tRNA\textsubscript{F} initiates polypeptide formation in a crude lens cell-free system. The NH\textsubscript{2}-terminal dipeptide contains the same amino acids as the N-terminus of \(\alpha\)-crystallin. The initiator properties of met-tRNA\textsubscript{F} 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 cortices were collected. Homogenization of the combined material was carried out in 0.008 M MgCl\textsubscript{2}, 0.025 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\(^\circ\). 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 \textit{E. coli} North-Holland Publishing Company – Amsterdam
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. 

<table>
<thead>
<tr>
<th>absorbancy at 260 nm</th>
<th>methionine acceptor activity (μmol/mg tRNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00-0.04</td>
<td>0.00-0.01</td>
</tr>
<tr>
<td>0.04-0.08</td>
<td>0.02-0.04</td>
</tr>
<tr>
<td>0.08-0.12</td>
<td>0.05-0.08</td>
</tr>
<tr>
<td>0.12-0.16</td>
<td>0.08-0.12</td>
</tr>
<tr>
<td>0.16-0.20</td>
<td>0.10-0.14</td>
</tr>
<tr>
<td>0.20-0.24</td>
<td>0.12-0.16</td>
</tr>
<tr>
<td>0.24-0.28</td>
<td>0.14-0.18</td>
</tr>
<tr>
<td>0.28-0.32</td>
<td>0.16-0.20</td>
</tr>
</tbody>
</table>

The three peaks of methionine acceptor activity are successively met-tRNAᵢ, met-tRNAᵢᵢ and met-tRNAᵢᵢᵢ.

Prepared according to the procedure of Muench Berg [20] and crude synthetase from rat liver using the procedure described by Takeishi et al. [21]. $^{14}$C-formate (specific activity 40 mCi/mole) was activated using a dialysed (NH₄)₂SO₄ fraction of pigeon liver supernatant [4], and radioactive aminoacyl-tRNA's were prepared from the dried fractions ($^{35}$S, specific activity 10–14 Ci/mole).

Preparation of the cell-free system

Fresh calf bovine eyes were obtained from the abattoir. 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 KCl. After homogenization the lysate was centrifuged at 10,000 g during 10 min and used in the incubation mixture.

Results and discussion

1 shows that chromatography of tRNA's from bovine eye lens on benzoylated DEAE-cellulose results in a clear separation of three fractions, namely tRNAᵢ¹, tRNAᵢᵢ¹, and tRNAᵢᵢᵢ¹ 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 also be 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-tRNAᵢ is low as compared to met-tRNAᵢᵢ and met-tRNAᵢᵢᵢ. However, a five-fold increase can be achieved after formylation of met-tRNAᵢ. 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 X 10⁻⁴ M inhibits initiation
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Fig. 2. Effect of Mg\(^{2+}\) concentration on the acylation reaction. The reaction mixture contained 0.6 μmoles ATP, 6 μmoles Tris-HCl, pH 7.4, 7.5 μmoles KCl, 1–2 × 10\(^{-3}\) μmoles \(^{14}\)C-methionine, 50–150 μg crude aminoacyl tRNA synthetase from rat liver or E. coli and 10 μg eye lens tRNA in a total volume of 150 μl. The incubation was for 15 min at 37°. Symbols represent the following: o—o—o : methionine acceptor activity of tRNA assayed with rat liver synthetase, •—•—• : methionine acceptor activity of tRNA assayed with E. coli synthetase, ••—••• : methionine acceptor activity of tRNA and tRNA assayed with rat liver synthetase.

Table 1
Effect of antibiotics on protein synthesis.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Met-tRNA(_I)</th>
<th>Met-tRNA(_II)</th>
<th>Met-tRNA(_III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100% (318 cpm)</td>
<td>100% (2145 cpm)</td>
<td>100% (1515 cpm)</td>
</tr>
<tr>
<td>A.T.A. 2 × 10(^{-4}) M</td>
<td>15%</td>
<td>60%</td>
<td>61%</td>
</tr>
<tr>
<td>cycloheximide 200 μg/ml</td>
<td>2%</td>
<td>2%</td>
<td>3%</td>
</tr>
<tr>
<td>chloramphenicol 100 μg/ml</td>
<td>105%</td>
<td>109%</td>
<td>95%</td>
</tr>
</tbody>
</table>

Reactions are described in the legend to fig. 3. Total volume of the incubation mixture was 30 μl, 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.

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 .RNA’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\(_A\) 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

<table>
<thead>
<tr>
<th>Ig₂⁺ conc. (nM)</th>
<th>Met-tRNA₁ (cpm)</th>
<th>F-Met-tRNA₁ (cpm)</th>
<th>Met-tRNA₁ (cpm)</th>
<th>Met-tRNA₁ (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ AUG</td>
<td>- AUG</td>
<td>+ AUG</td>
<td>- AUG</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>50</td>
<td>305</td>
<td>290</td>
</tr>
<tr>
<td>8</td>
<td>365</td>
<td>40</td>
<td>630</td>
<td>300</td>
</tr>
<tr>
<td>2</td>
<td>2100</td>
<td>45</td>
<td>2230</td>
<td>490</td>
</tr>
<tr>
<td>5</td>
<td>3315</td>
<td>215</td>
<td>4630</td>
<td>910</td>
</tr>
<tr>
<td>0</td>
<td>4010</td>
<td>685</td>
<td>4965</td>
<td>1600</td>
</tr>
</tbody>
</table>

Binding reaction contained in a 40 µl volume: 1.0 A₂₆₀ units of washed E. coli ribosomes, 0.05 A₂₆₀ units of ApUpG, 0.1 M MSCI, pH 7.5, 0.05 M KCl, 0.005 M β-mercaptoethanol, 0.5 pmole ³⁵S met-tRNA₁, 0.5 pmole Formyl-³⁵S-met-tRNA₁, 0.5 µmole ³⁵S-met-tRNA₁ and 0.5 pmole ³⁵S-met-tRNA₁ (1 pmole = 20,000 cpm). The mixture was incubated for 8 min at 37°C. 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—asp. (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 NH₂-terminal methionine is removed shorter or later after initiation. If this situation would occur in α-crystallin synthesis, an NH₂-terminal sequence met—met—asp has to be expected. In that case we should have found F-met—met—(asp) instead of F-met—asp. As the latter peptide is found as an NH₂-terminal

Fig. 4. Pronase digestion of formyl-³⁵S-methionyl product. After 20 min incubation at 37°C (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 NH₄HCO₃ and 2 mM CaCl₂ containing pronase at 0.2 mg/ml. After incubation at 37°C 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°C 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 NH₂-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 NH₂-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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References