

Calf Crystallin Synthesis in Frog Cells: The Translation of Lens-Cell 14S RNA in Oocytes

(messenger RNA/initiation factors/protein synthesis/acetyl methionine)

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ABSTRACT 14S RNA isolated from calf-lens polyribosomes was injected into oocytes of the frog *Xenopus laevis*. Oocytes injected with 14S RNA and buffer contained a protein resembling the A2 chain of calf α -crystallin; oocytes injected with buffer alone contained no crystallin-like material. α A2 crystallin polypeptides were identified by various criteria: urea-gel electrophoresis under acidic and basic conditions, gel electrophoresis in sodium dodecyl sulfate, N-terminal analysis, and paper chromatography of methionine-containing tryptic peptides.

It is concluded that when it is injected into a living frog oocyte, the 14S RNA from lens tissue is reasonably stable and has the properties of an α A2 crystallin messenger. The messenger requires no lens cell-specific components for translation within the oocyte, and the translational machinery of the frog cell will accept messenger RNA from a totally different cell type from another species.

The A2 chains of α -crystallin extracted from lens tissue possess an acetylated N-terminal methionine residue; the N-terminal methionine of α A2 chains derived from frog oocytes injected with 14S RNA was also acetylated.

Several interesting questions in developmental biology can be studied by combination of messenger RNA from one kind of cell with the translational apparatus of another cell type. Such experiments can provide proof of the identity of a messenger RNA, and can yield information regarding tissue-specific requirements and species- and cell-type specificities of the translation process.

When 9S RNA from rabbit reticulocytes is injected into frog oocytes, hemoglobin is synthesized, showing the absence of any requirement for tissue-specific factors and the presence within the oocyte of nonspecific translational apparatus (1). This paper describes the synthesis of A2 chains of α -crystallin in oocytes injected with 14S RNA from calf-lens tissue. For translation of the crystallin messenger (2, 3), there is no requirement for tissue-specific factors, and some, at least of the translational apparatus within the oocyte, are neither cell- nor species-specific. Similar conclusions were reached by Lane, Marbaix, and Gurdon (1) with rabbit hemoglobin messenger.

As in α A2 chains extracted from lens tissue (4), chains from oocytes injected with 14S RNA possess an N-terminal acetylated methionine residue. This observation is discussed in terms of the possibility that the protein-synthesizing machinery of different tissues possesses an acetylating mechanism that is able to recognize a certain amino acid sequence.

METHODS

Handling of Oocytes. The injection procedure and culture medium were as described (1), except that batches of 40 oocytes were incubated at 21° in medium containing [³⁵S]-methionine (26 Ci/mmol; 0.5 mCi/ml). 14S RNA was dissolved in the injection medium (88 mM NaCl-1.0 mM KCl-15 mM Tris·HCl, pH 7.6) at a concentration of 2 mg/ml.

Isolation of 14S RNA from Calf-Lens Tissue. Polyribosomes from calf lens were isolated as described (5), except that cortical, but not epithelial, tissue was used for the preparation. Polyribosomes were suspended (6) in a medium containing 6% (w/w) sucrose, 0.05 M Tris·HCl, and 1% sodium dodecyl sulfate (SDS). The final pH was 7.4. After incubation at 37° for 5 min, the polyribosomes were diluted twice with the same medium lacking SDS. 10-25 ml of sample, containing about 3 mg/ml of polysomal material, was applied to an exponential 8-28% sucrose gradient. After it was overlaid with 150 ml of buffer, the gradient was centrifuged at 50,000 rpm for 15 hr at 2° in a Bxxx IEC rotor. The gradient profile was monitored at 260 nm with a Gilford spectrophotometer, adapted to a 2-mm flow cell. All sucrose solutions used were boiled with 0.02% diethylpyrocarbonate for 30 min. The fraction corresponding to 14S RNA was precipitated by addition of 0.1 volume of 2 M potassium acetate, pH 5.0, and 2.5 volumes of cold ethanol; the solution was allowed to stand for 16 hr at -25°. RNA was pelleted and dissolved in the injection buffer.

Homogenization of Oocytes. Thawed samples of oocytes were homogenized in 10 μ l per oocyte of a medium containing 0.05 M Tris·glycine, 0.08 M KCl, 0.05 mM methionine, and calf-lens α -crystallin (100 μ g/ml). The final pH was 8.9. The homogenate was centrifuged at 3500 \times g for 15 min at 4°. 0.04 Volumes of 0.2 M EDTA and 0.1 volume of 0.2 mg/ml pancreatic ribonuclease were added and, after incubation at 37° for 15 min, 0.2 volumes of 50% Cl₃CCOOH was added; the precipitated protein was washed four times with 5% Cl₃CCOOH, once with ethanol, once with ethanol-ether 1:1, and once with ether. The material was dried at room temperature and subjected to electrophoretic analysis.

Acrylamide Gel Electrophoresis. Samples containing about 75,000 cpm of [³⁵S]methionine-labeled material were mixed with 25 μ g of marker α -crystallin, and were electrophoresed (7) in acidic or basic urea gels. After staining and destaining, the gels were handled as described below. For SDS-gel electrophoresis, the precipitates were dissolved in a solution containing 10 mM sodium phosphate, pH 7.0-1% SDS-1%

Abbreviation: SDS, sodium dodecyl sulfate.

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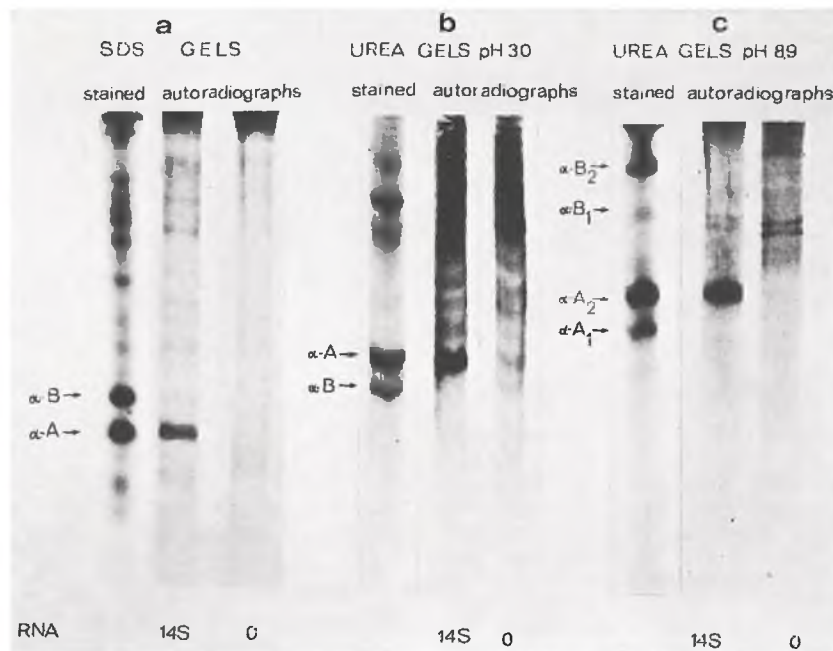


FIG. 1. Oocytes were injected and homogenized with marker α -crystallin; the supernatant was analyzed on polyacrylamide gels.

2-mercaptoethanol, and were heated at 100° for 3 min. Each sample contained about 75,000 cpm of [³⁵S]methionine, and electrophoresis was on 12.5% acrylamide gels, prepared according to the method of Weber and Osborn (8), in 0.6 × 0.8-cm glass tubes, at 5 mA/gel for 12 hr. The gels were stained with Coomassie blue, sliced, dried, and autoradiographed for 16 hr with Kodak Royal X-omat type RHP x-ray film.

Tryptic Digestion of the αA_2 Polypeptide. αA_2 chains were separated from the majority of the oocyte proteins by gel electrophoresis on basic urea gels. The gel segment containing the αA_2 band was cut out, minced, dialyzed against 5 mM of 2-mercaptoethanol, and, after filtration to remove the gel, the solution was lyophilized. Part of the material so obtained was aminoethylated by the method of Raftery and Cole (9). After precipitation in 15% Cl₃CCOOH and removal of the acid by acetone washing, the material was dissolved in 0.1 M NH₄HCO₃ and digested with trypsin (1:50, w/w) for 5 hr at 37°. The resulting peptides were lyophilized, dissolved in water, and subjected to descending paper chromatography on Whatman 3 MM paper eluted with butanol-acetic acid-pyridine-water 60:12:48:40. Tryptic peptides from [³⁵S]-methionine-labeled αA_2 crystallin prepared by *in vitro* incubation of a lysate from calf lens were used as markers. These markers have the same chromatographic behavior as do the native methionine-containing peptides of αA_2 (unpublished results). Chromatograms were dried and cut into 1-cm strips, which were counted in a liquid scintillation counter.

Pronase Digestion of the αA_2 Polypeptide. Material from the αA_2 band was digested (10) in 0.1 M NH₄HCO₃-1 mM CaCl₂ for 6 hr at 37°. The digest was lyophilized, dissolved in distilled water, and electrophoresed on Whatman 3 MM paper in acetic acid-pyridine-water 6:200:794 (pH 6.5) for 2 hr at 45 V/cm. Radioactivity was determined as described above for paper chromatography. Reference peptides were stained with platinum iodide (11).

RESULTS

Oocytes were injected with calf-lens 14S RNA dissolved in injection medium, and were frozen after 18 hr of incubation with [³⁵S]methionine. Control oocytes were treated similarly, except that the 14S RNA was omitted from the injection mixture. Newly synthesized proteins from both batches of oocytes were then analyzed for their content of α -crystallin-like material.

Identification of whole α -crystallin components

The radioactive oocyte proteins were first analyzed by SDS-gel electrophoresis, a technique known to resolve α -crystallin into two components, αA and αB , having molecular weights of 19,000 and 22,000, respectively (12). Fig. 1a shows that the presence of 14S RNA in the injection mixture is associated with the formation of radioactive molecules that coelectrophorese with the αA component of added marker α -crystallin.

Acidic urea gels also separate the acidic αA component from the basic αB component (7). Fig 1b confirms that the presence of 14S RNA in the injection mixture is associated with the formation of oocyte-derived material that coelectrophoreses with the αA component of marker α -crystallin. Basic urea gels resolve α -crystallin into its four constituent polypeptide chains, αA_1 , αA_2 , αB_1 , and αB_2 (7). [³⁵S]-Methionine-labeled material from oocytes injected with 14S RNA analyzed on such a basic urea gel contained labeled material that coelectrophoresed with the A_2 chain of marker α -crystallin. No radioactive material preferentially migrates to the αA_1 , αB_1 , or αB_2 regions of the gel. If oocytes injected with 14S RNA and controls are labeled with a mixture of tritiated aminoacids (lysine, leucine, and histidine), the three types of gel analysis again reveal labeled αA_2 crystallin chains in cells injected with messenger, but not in control cells.

The A_2 chain of calf-lens α -crystallin normally contains one acetylated N-terminal methionine and one internal methionine residue (4). αA_2 chains isolated from basic urea gels were

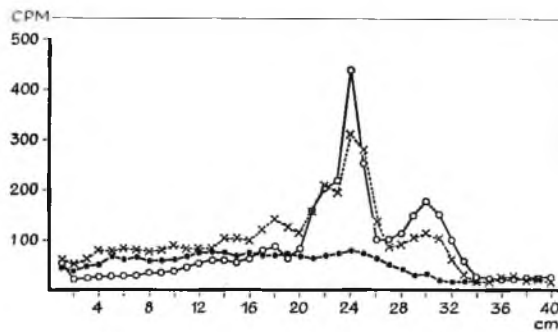


FIG. 2. Identification of the methionine-containing tryptic peptides of α A2 chains by paper chromatography. X---X, peptides derived from oocytes injected with 14S RNA; O---O, peptides derived from lens; ●---●, peptides derived from oocytes injected with buffer only.

digested with trypsin, and the resulting peptides were chromatographed on paper. Marker peptides derived from a tryptic digest of [35 S]methionine-labeled α A2 chains from calf lens, were run on an adjacent strip of paper. Fig. 2 shows that the methionine peptides from oocyte-derived α A2 material have the same chromatographic mobility as do the reference peptides derived from calf-lens α A2 material. The fast-moving component shown in Fig. 2 is the N-terminal peptide; the slow-moving component is the internal peptide. The slow-moving peptide is partially resolved into a major and a minor component, the minor component representing the oxidized form of this methionine peptide: the two oxidation states of the fast-moving component are not resolved by this chromatographic solvent. Fig. 2 shows that the internal and N-terminal peptides from oocyte-derived material are not pres-

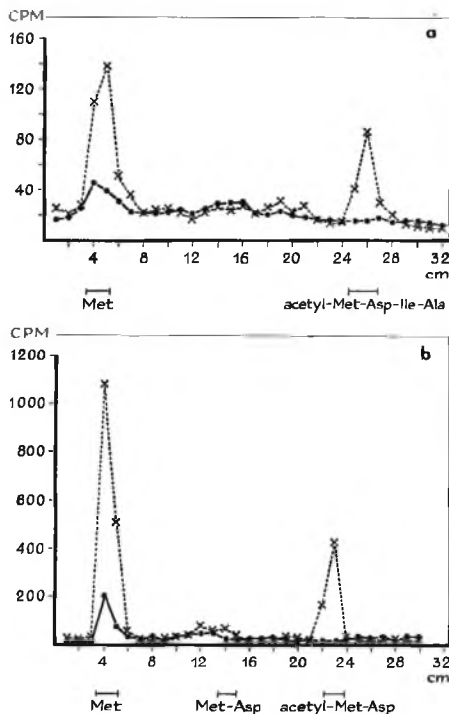


FIG. 3 (a). Identification of the N-terminal tetrapeptide of α A2 by paper electrophoresis. (b). Identification of the N-terminal dipeptide of α A2 by paper electrophoresis. X---X, peptides derived from oocytes injected with 14S RNA; ●---●, peptides derived from oocytes injected with buffer only.

ent in equal amounts; this inequality can also be seen in the [35 S]methionine-labeled α A2 reference peptides, and it is probably the result of incomplete digestion. The N-terminal peptide of α A2 chains from calf lens is acetylated (10); the similarity in chromatographic behavior between the N-terminal peptides of oocyte-derived and lens-derived α A2 chains suggests, but does not prove, that the N-terminal methionine residue is also acetylated in chains made in the oocyte. The N-terminal sequence of all α -crystallin polypeptides is N-acetyl-Met-Asp-Ile-Ala; subtilisin digestion releases a peptide of this sequence (13). Pronase digestion releases the dipeptide of this sequence (13). Fig. 3a shows the result of electrophoresis of the products of subtilisin digestion of [35 S]methionine-labeled α A2 chains from oocytes; Fig. 3b shows the results obtained from digestion with Pronase. Synthetic reference peptides were electrophoresed as standards. The results show that the N-terminal peptide from oocyte-derived α A2 chains is blocked, for in the case of Pronase digestion the electrophoretic conditions used are capable of resolving the free and blocked N-terminal peptides. To exclude the possibility that the blocking agent is a formyl group, one sample of the N-terminal dipeptide was heated with 0.5 N HCl for 30 min at 90° (14); there was no shift of radioactivity to the Met-Asp region of the chromatogram. We conclude, therefore, that frog oocytes programmed with 14S RNA from calf-lens synthesize material that is extremely similar, if not identical, to A2 chains of calf-lens α -crystallin, even to the extent that the oocyte-derived α A2 chains are N-acetylated.

DISCUSSION

Our results show that 14S RNA from calf lens, when injected into a frog oocyte, is not only spared from rapid degradation but is also translated, giving rise to material closely resembling calf α A2 crystallin.

α -Crystallin from calf lens is composed of two acidic polypeptide chains, α A1 and α A2, and two basic chains, α B1 and α B2. The material from oocytes injected with 14S RNA was shown, by SDS-gel electrophoresis, to have a molecular weight identical to that of the acidic chains of α -crystallin. Gel electrophoresis in acidic urea showed that the oocyte-derived material yielded molecules whose overall charge was equal to that of A chains from calf-lens α -crystallin. Gel electrophoresis in basic urea showed that oocytes injected with 14S RNA contain molecules of the same electrophoretic mobility as marker calf-lens α A2 chains. Paper chromatography of methionine-containing peptides showed that those from oocyte-derived α A2 chains were indistinguishable from those of lens-derived α A2 chains. Subtilisin and Pronase treatment of α A2 chains from oocytes released methionine peptides that were electrophoretically identical to acetyl-Met-Asp-Ile-Ala and acetyl-Met-Asp, respectively; these peptides correspond to the N-terminal sequence of α -crystallin (10, 13). As judged by all these analytical criteria, control oocytes contained no detectable amounts of crystallin-like substances.

Oocytes injected with 14S RNA contain, therefore, a substance that is extremely similar to A2 chains of calf α -crystallin; however, the criteria used would not necessarily reveal subtle differences in sequence (i.e., we have not yet measured the fidelity of translation).

The results indicate that the 14S RNA fraction from calf

§ Experiments are in progress to try to show that all tryptic peptides from the newly synthesized polypeptides are identical to the tryptic peptides from the native α A₂ chain.

lens contains messengers coding for α A2 crystallin chains, in accord with information obtained from cell-free extracts (2, 3). However, the experiments described do not prove that oocytes injected with 14S RNA synthesize calf α A2 crystallin chains; it is conceivable that the 14S RNA elicits the synthesis of frog crystallin chains. However, this possibility is virtually ruled out by data obtained for the analogous situation of oocytes injected with rabbit reticulocyte 9S RNA (1) (15) (Marbaix, G. & Lane, C. D., paper in preparation).

Our results also contribute to the question of the species and cell-type specificities, and requirements of the translation process. Since calf-lens crystallin messenger RNA is successfully translated in frog oocytes, we can conclude that the components required to translate calf-lens α A2 crystallin messenger RNA are present in cells as unrelated as calf-lens cortex cells and frog oocytes. Thus, if messenger-specific components are required for the translation of crystallin messenger, then such components are present and available in frog oocytes. If it is assumed that such factors do exist in oocytes, then it is clear that their presence cannot be the only phenomenon determining the appearance of cell-type specific proteins during cell differentiation. The results strongly suggest, but do not prove, that the translational machinery of the frog oocyte is not cell-type specific:

This observation, showing that no additional factors are required for the translation of exogenous mRNA, is consistent with other experiments performed with whole oocytes (1) and with results obtained from various crude cell-free systems (2, 3, 16-18). Evidence for tissue specificity has only been found with purified cell-free extracts derived from terminally differentiated tissues (20, 21). These experimental results are not necessarily inconsistent, for it is possible that tissue specificity is masked in crude cell-free systems, and that the oocytes do not have such restrictive translational requirements as do the cells of terminally differentiated tissues.

The A2 chains of α -crystallin are N-terminally acetylated (4). Our results show that oocytes injected with 14S RNA give rise to N-acetylated α A2 crystallin chains. The same is true of 14S RNA-directed α A2 crystallin synthesis in a reticulocyte lysate (paper in preparation). Three possible acetylating mechanisms may be considered.

(i) The 14S messenger RNA, which has an estimated molecular weight of 360,000 (2), is large enough to code for two polypeptide chains, each of 20,000 molecular weight. One of these two chains may be an acetylating enzyme. Or, the α A2 polypeptide may itself have acetylating properties.

(ii) The 14S messenger may require acetyl-Met-tRNA_f^{Met} for initiation.

(iii) Acetylation takes place after initiation with Met-tRNA_f^{Met}, and is determined by the N-terminal sequence.

The first possibility is rendered unlikely by the observation that 10S RNA from lens tissue directs the synthesis of N-acetylated α -crystallin B chains in a reticulocyte cell-free system (paper in preparation), while the aminoacid sequences of the B chains are, with the exception of the N-termini, totally different from those of the A chains. The second possibility is unlikely, for no acetylated Met-tRNA_f^{Met} can be detected in lens tissue (unpublished results) or in reticulocytes (19).

The most obvious explanation is, therefore, that the protein synthesizing machinery of different tissues, from different species, possesses an acetylating mechanism that is able to recognize and acetylate a certain aminoacid sequence. One

may speculate that all eukaryotic cells possess an acetylating mechanism, ribosome-bound or free in the cytoplasm, of this general nature.

The observed disparity between the size of the 14S message and the newly synthesized polypeptide may be explained in several ways.

(i) The 14S mRNA contain rather long untranslated regions. However, the low AMP content (6) makes the occurrence of long A-rich regions unlikely.

(ii) The 14S mRNA may be bicistronic.

(iii) The 14S mRNA may code for a longer precursor molecule, which is proteolytically trimmed to yield α A₂.

Further experiments are necessary to clarify this disparity. The other lens messenger fraction, characterized by a sedimentation coefficient of 10 S, and with an average molecular weight of 260,000, codes for crystallin polypeptide chains of higher molecular weight than α A₂ (3).

Our results also show that calf-lens α A₂ crystallin chains are stable, at all stages of assembly, in frog oocytes. Moreover, the translation of the crystallin messenger and the stability of the products formed lend support to the idea that the oocyte system may prove to be a generally useful microassay for eukaryotic mRNA molecules.

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Synthesis of Lens Protein *in vitro*

Role of Methionyl-tRNAs in the Synthesis of Calf-Lens α -Crystallin

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The main polypeptide chain of the lens protein α -crystallin (αA_2) is N-terminally acetylated and contains two methionine residues. The chain is initiated by the incorporation of a methionine residue exclusively donated by methionyl-tRNA^{fMet}.

This N-terminal methionine is not removed during polypeptide chain synthesis. The internal methionine residue is donated exclusively by one of the methionyl-tRNA^{Met} species. When lens messenger RNA coding for the αA_2 chain is translated in a reticulocyte cell-free system, the newly synthesized polypeptide chain carries an acetylated methionine in the N-terminal position.

A specific initiator tRNA is known to be involved in peptide-chain initiation on 80-S ribosomes in eukaryotic cells [1–6]. In general the following hypothesis about the mechanism of the initiation of protein biosynthesis is accepted: Protein synthesis starts with a methionine residue, donated by a specific initiator tRNA, whilst donation of methionine residues for internal positions in the polypeptide chain occurs by either of the tRNA^{Met} species which differ in a number of properties [7]. Also, the N-terminal methionine residue, as a rule, is removed during polypeptide chain elongation.

In bacterial systems protein synthesis starts with formylated Met-tRNA^{fMet} [8]. However in eukaryotic cells acylation of the methionyl-tRNA^{fMet} during the initiation process either *in vivo* or *in vitro* could not be demonstrated [9].

The eye lens is an interesting system for the study of the mechanism of initiation and the role of the different methionyl-tRNAs because of a few particular features. Firstly, α -crystallin, the most intensively studied lens protein, contains two methionine residues per polypeptide chain: one N-terminally and one in internal position. Secondly, the N-terminal methionine residue is acetylated.

Since messenger RNA coding for α -crystallin is at our disposal [10] we were able to compare the initiation mechanism in different cell-free systems.

Abbreviations. Methionyl-tRNA^{fMet}, the charged species of tRNA corresponding to methionine, which can be formylated by the *E. coli* transformylase; methionyl-tRNA^{Met}, the charged species of tRNA corresponding to methionine, which cannot be formylated. In lens tissue two Met-tRNA^{Met} species occur assigned as tRNA₁^{Met} and tRNA₂^{Met} eluted consecutively from the benzoylated DEAE-cellulose column.

The first feature enabled us to examine the specificity of the methionyl tRNAs in the synthesis of α -crystallin. Concerning the question of N-terminal acetylation some confusion has arisen during the last decade: is acetylation involved in the initiation mechanism in eukaryotic cells [11–13] or does it act independently of the initiation process [14]? In the present paper an attempt has been made to shed some light on this problem.

All experiments described here were performed with A_2 , the major polypeptide chain of α -crystallin.

EXPERIMENTAL PROCEDURE

Materials

L-[³⁵S]Methionine (15–20 Ci/mmol) was obtained from the Radiochemical Centre (Amersham, England).

Trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone) was a product purchased from Worthington Biochemical Corporation, pronase from *Streptomyces griseus* was delivered by Serva (Germany). Carboxypeptidase A was suspended in toluene and had been treated with diisopropylphosphofluoridate by the supplier (Sigma). Sunitilisin was obtained from Nutritional Biochemicals Co. (Cleveland, Ohio). Ethylenimine was delivered by Fluka.

Preparation of Lens Transfer RNA

Lens tRNA was isolated by phenol-dodecylsulfate extraction. After separation of the three different methionine tRNA species by benzoylated DEAE-cellulose column chromatography as describ-

ed previously [7] tRNA^{Met} was charged with the aid of crude synthetase from *Escherichia coli* to avoid cross contamination with tRNA^{Met} as *E. coli* synthetase is not able to charge tRNA^{Met}. The two tRNA^{Met} fractions were charged with crude liver synthetase. The [³⁵S]methionyl-tRNAs were dissolved in 5 mM potassium acetate pH 5.0 and stored at -20 °C.

The Cell-Free System

The epithelial part and outer cortex from calf lenses were collected as soon as possible after death of the animals. One volume of cold deionized water was added and homogenization was carried out with a tight fitting teflon homogenizer. After centrifugation at 15000×g for 10 min the lysate was frozen in liquid nitrogen and stored at -70 °C. Synthesis of lens protein *in vitro* was carried out in incubation mixtures containing per ml 1 μmol ATP, 0.5 μmol GTP, 5.0 μmol 2-mercaptoethanol, 10 μmol creatine phosphate, 50 μg creatine phosphokinase, 50 μmol Tris-HCl pH 7.4, 50 μmol KCl, 3 μmol magnesium acetate and 0.1 μmol of all twenty amino acids. Dependent on the type of experiment either [³⁵S]methionine (50 μCi) or 1.5×10⁶ counts/min of one of the [³⁵S]methionyl-tRNA fractions (approx. 40000 counts×min⁻¹×μg⁻¹ tRNA) was added.

Incubations were carried out at 30 °C for 60 min, pancreatic ribonuclease (100 μg/ml) and EDTA (0.03 M) were added and incubation was continued for 15 min. The samples were precipitated with trichloroacetic acid and centrifuged. The precipitate was washed three times with 5% trichloroacetic acid and lyophilized.

For translation of lens messenger RNA in a reticulocyte cell-free system the incubation conditions were as described previously [10] except that [³⁵S]methionine was used instead of formyl-[³⁵S]-methionyl-tRNA^{Met}.

Isolation of the αA₂ Chain

Separation of the αA₂ chain from the other proteins in the lens system or from globin in the reticulocyte system was carried out on polyacrylamide gels at basic pH in the presence of 6 M urea, as described elsewhere [15]. Per gel, 4–5 mg of lyophilized protein dissolved in 200 μl 6 M urea-Tris-EDTA-boric acid buffer pH 8.9 was applied. The gels (1.2×9 cm) were run overnight at 5 mA per gel. Thereafter thin longitudinal slices were stained with amido black and destained electrophoretically. The αA₂ band was cut out from the unstained part of the gel, fragmented and dialyzed against water for 24 h. The gel was removed by filtration. The protein solution was lyophilized and the material was aminoethylated with ethylenimine

in 7 M urea using the method of Raftery and Cole [16]. The protein was purified by gel filtration on a Sephadex G-25 column in 0.5% formic acid and lyophilized.

Enzymic Digestion

Digestion of the material from the αA₂ bands was performed with trypsin, subtilisin, pronase and carboxypeptidase A.

Tryptic digestion was carried out at pH 8.0 in 0.1 M ammonium bicarbonate (1 mg trypsin per 50 mg protein) for 5 h at 37 °C. For identification of the methionine-containing peptides aliquots of the digested material were subjected to paper chromatography on Whatman 3-MM paper and eluted with a mixture of butanol-acetic acid-pyridine-water (60:12:48:40, v/v/v/v) for 16 h.

Digestion of the αA₂ chain with subtilisin releases the N-terminally-acetylated tetrapeptide (Ac-Met-Asp-Ile-Ala).

The digestion was carried out following the procedure of Hoenders *et al.* [17]. As marker the N-terminal tetrapeptide prepared from native α-crystallin and purified by Dowex 50 (H⁺) chromatography was used. Pronase and carboxypeptidase A digestions were performed as described by Hoenders *et al.* [18].

Analysis of the products after subtilisin, pronase and carboxypeptidase A digestion were carried out by electrophoresis on Whatmann 3-MM paper equilibrated in acetic acid-pyridine-water (6:200:794, v/v/v) pH 6.5 for 2 h at 45 V/cm. Electropherograms and chromatograms were cut into 1-cm strips and the radioactivity was counted in a scintillation counter using a toluene-based scintillator. Reference peptides were stained using platinum iodide [19].

RESULTS AND DISCUSSION

The Origin of the N-Terminal Methionine of α-Crystallin

When [³⁵S]methionyl-tRNA^{Met} derived from lens tissue is incubated with a crude lens cell-free system whereafter pronase digestion is performed, the sequence Ac-[³⁵S]Met-Asp can be identified in newly formed α-crystallin polypeptides (Fig. 1A). This sequence is known to be the N-terminal dipeptide of native α-crystallin chains [18].

Label in the Ac-Met-Asp region is only observed after incubation with [³⁵S]Met-tRNA^{Met}; no radioactivity at all can be detected if the incubation is carried out with one of the Met-tRNA^{Met} species derived from lens tissue. To ascertain the identity of the dipeptide the material at the position of Ac-Met-Asp was eluted from the paper and electrophoresed after digestion with carboxypeptidase A.

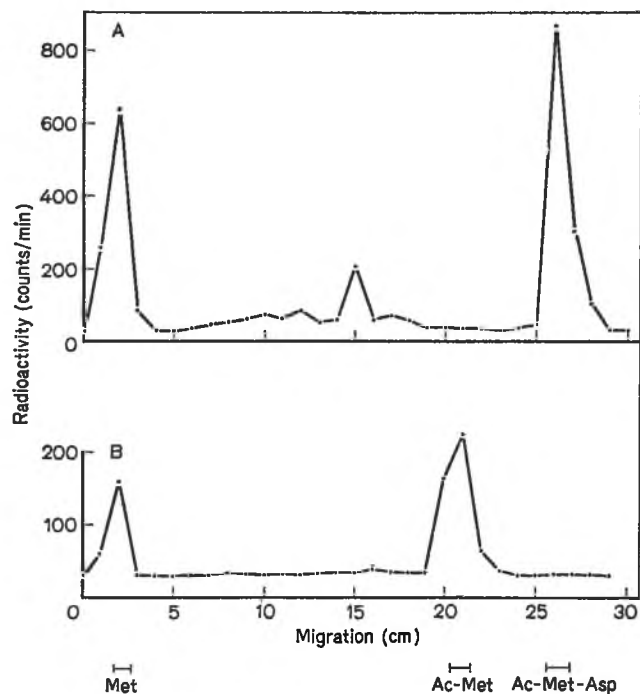


Fig. 1. Identification of NH_2 -blocked peptides after pronase digestion. [^{35}S]Methionine-labelled protein from lens lysate was digested with pronase. After incubation at 37°C for 6 h a suspension of 1 ml Dowex-50 resin in H^+ form was added. The water layer containing the blocked peptides was lyophilized and analyzed by paper electrophoresis at pH 6.5 (A). The radioactivity migrating together with unlabelled acetyl-methionyl-aspartic acid was eluted from the paper and treated with carboxypeptidase A. After incubation the product was again analyzed by paper electrophoresis (B)

This treatment caused a shift of the radioactivity to the position of acetyl-methionine (Fig. 1B). From these results and our previous findings that fMet-tRNA^{fMet} gives rise to the sequence fMet-Asp in α -crystallin [7] we conclude that the N-terminal methionine of α -crystallin is donated exclusively by Met-tRNA^{fMet}.

Transfer of Methionine from Different Methionyl-tRNA Species into the Lens αA_2 Polypeptide Chains

In order to get an insight into the specificity of the three methionyl-tRNA species isolated from the cytoplasm of bovine lens [7], we examined the two methionine-containing tryptic peptides of the A_2 chain of α -crystallin. After aminoethylation of the αA_2 polypeptide trypsin releases two methionine-containing peptides [20, 21] which can be separated by paper chromatography.

It appeared that during handling the methionine residues in the tryptic peptides are sulfoxidized, partly

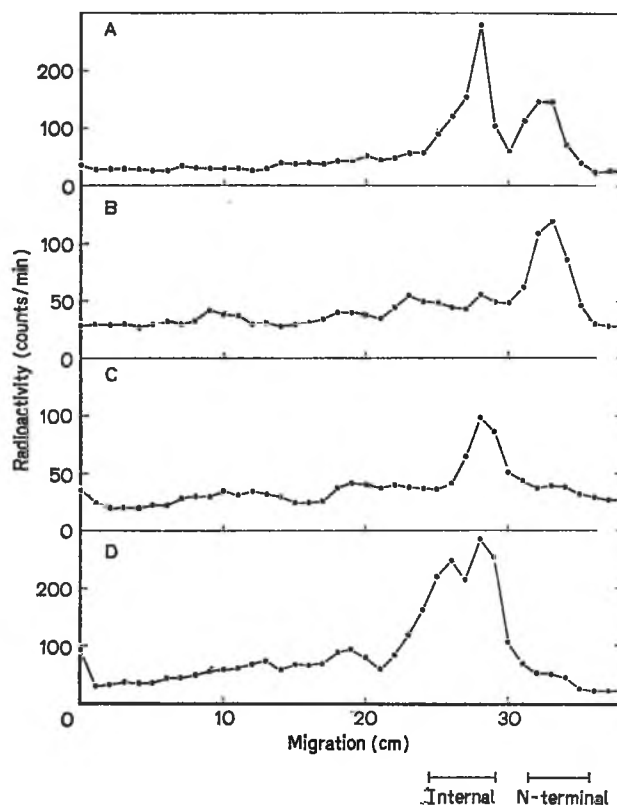


Fig. 2. Paper chromatography of the methionine-containing tryptic peptides of the αA_2 chains. (A) Peptides obtained after [^{35}S]methionine labeling; (B) peptides obtained after labeling with [^{35}S]Met-tRNA^{fMet}; (C) peptides obtained after labeling with [^{35}S]Met-tRNA₁^{Met}; (D) peptides obtained after labeling with [^{35}S]Met-tRNA₂^{Met}

in the internal and completely in the N-terminal peptides. As sulfoxidation brings about a lowering of the R_F value of the peptides two spots for the internal and one for the N-terminal peptide are found. Fig. 2 shows the distribution of label on chromatography of the tryptic peptides obtained after incubation with [^{35}S]methionine (A); [^{35}S]Met-tRNA^{fMet} (B); [^{35}S]Met-tRNA₁^{Met} (C), and [^{35}S]Met-tRNA₂^{Met} (D). The labelled spots in (A) have the same chromatographic behaviour as the native methionine-containing tryptic peptides of αA_2 chains.

The results depicted in (B) prove once more that the methionine residue present in N-terminal position is donated exclusively by Met-tRNA^{fMet}. Moreover it is demonstrated that tRNA^{Met} supplies the methionine only to the internal peptide (C and D).

As these observations are entirely in accord with previous findings concerning the initiation mechanism in other mammalian systems [4—6, 9], the conclusion is justified that the initiation mechanism of protein synthesis in the lens functions in exactly the same way as reported for other tissues. That the N-terminal

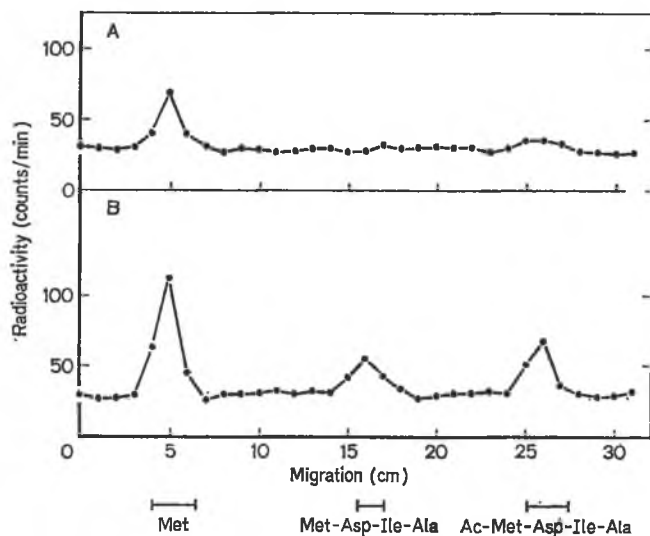


Fig. 3. Identification of the N-terminal tetrapeptide of the αA_2 chain by paper electrophoresis. The incubation was carried out in a reticulocyte lysate for 60 min at 37 °C as described previously [10]. Thereafter carrier α -crystallin was added to the mixture, the αA_2 chain was separated by polyacrylamide gel electrophoresis, isolated and subjected to digestion with subtilisin and analyzed by high-voltage electrophoresis at pH 6.5 as described in Methods. (A) Incubation minus 14-S lens mRNA; (B) incubation in the presence of 14-S mRNA

methionine of the α -crystallin chains is not removed at any stage of the process of biosynthesis was shown by incorporation experiments with fMet-tRNA^{fMet} [7]. These studies revealed that fMet-Asp was the only N-terminal dipeptide.

We mentioned earlier that in the native state the N-terminal methionine is acetylated. Until now all attempts to find an acetylated methionyl-tRNA in lens tissue were unsuccessful. To examine whether the methionyl-tRNA^{fMet} bound to the ribosomes was modified during initiation we have used sparsomycin which is known to restrict the growth of newly initiated polypeptide chains. After incubation in the presence of 40 μ M sparsomycin neither Ac-Met-tRNA nor Ac-Met-Asp-tRNA bound to the ribosomes could be detected. In contrast Met-tRNA^{fMet} was found exclusively. These observations render the possibility that initiation of α -crystallin requires an Ac-Met-tRNA species very unlikely.

Occurrence of a N-Terminal-Acetylating Mechanism in Rabbit Reticulocytes

We were able to show that translation of lens messenger RNA in a rabbit reticulocyte cell-free lysate results in a newly synthesized peptide chain with the same properties as the native αA_2 chain

[10]. In these experiments f-[³⁵S]Met-tRNA^{fMet} derived from lens was used as radioactive precursor.

To our surprise in subsequent experiments we observed that after incubation of a reticulocyte lysate with [³⁵S]methionine in the presence of 14-S lens mRNA the newly synthesized αA_2 chain carried an acetyl group in the N-terminal position. This was established by several analytical procedures. Fig. 3 shows that the presence of the 14-S lens mRNA in the incubation mixture is accompanied by the formation of a polypeptide from which subtilisin releases a compound with an identical electrophoretic behaviour to the native N-terminal peptide Ac-Met-Asp-Ile-Ala of α -crystallin (Fig. 3B). Radioactivity is also found in the position of the non-acetylated tetrapeptide. If this label represents Met-Asp-Ile-Ala, which awaits further identification, this lends support to the assumption that acetylation occurs after initiation. Upon incubation without 14-S mRNA no radioactivity is found in the region of the tetrapeptides (Fig. 3A).

A comparable result can be obtained after digestion with trypsin. In that case an identical chromatographic pattern, as shown in Fig. 2A, is observed. Furthermore after digestion of the N-terminal tryptic peptide with pronase the acetylated dipeptide is obtained.

Other studies in our laboratory revealed that translation of lens messenger RNA in frog oocytes also results in N-terminal acetylation of the αA_2 chain [22].

In the reticulocyte system much work has been carried out to examine the fate of Met-tRNA^{fMet} during the process of initiation of gobin synthesis [4, 5, 7, 23]. The initiation process *per se* appears to be quite similar for different proteins synthesized on 80-S ribosomes including lens crystallins. The available data suggest that N-terminal acetylation is not an integral part of the initiation mechanism. The reason why α -crystallin chains are acetylated in contrast to many other proteins might be that the acetylating mechanism is only operative when it recognizes distinct features of a polypeptide chain like a certain amino acid sequence or secondary structure. On the other hand the universality of the acetylation mechanism is strongly suggested by the finding that α -crystallin chains are acetylated in totally different systems like calf lens lysate, rabbit reticulocyte lysate and frog oocytes.

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