Synthesis of Lens Protein *in vitro*

N-Terminal Acetylation of α-Crystallin

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N-terminal acetylation of the lens protein α-crystallin has been studied in a lens cell-free system. This system is capable of synthesizing *de novo* α-crystallin, a protein with acetylmethionine as N-terminus. When [^{35}S]Met-tRNA^{fMet} is used as radioactive precursor only the α-crystallin chains are labeled in the N-terminal position. This phenomenon enables the study of the process of acetylation. Newly synthesized polypeptides could be separated according to chain length. Electrophoretic analysis of thermolytic peptides revealed that acetylation of the N-terminus occurs while the chain is still on the ribosome.

It is concluded that the N-terminal acetylation takes place after chain initiation and before completion of the polypeptide chain.

Bacterial as well as eukaryotic systems are capable of acetylating a number of their proteins. It would therefore seem that N-terminal acetylation of proteins is a general phenomenon in living cells.

However, studies on acetylation in relation to other events in protein biosynthesis have revealed neither the exact role nor the very moment of acetylation [1,2]. Together with the elucidation of the primary structure of proteins the list of *N*-acetylated proteins is growing (see discussion Table 2). In earlier studies on the initiation of protein biosynthesis in eukaryotes it was suggested that synthesis of some proteins may start with an acetylated amino acid, such as *N*-acetylvaline for rabbit globin [3], *N*-acetylglycine for ovalbumin [4] and *N*-acetylserine for histones [5]. However, since it is generally accepted now that initiation of protein synthesis in prokaryotes as well as in eukaryotes takes place according to a universal mechanism in which *N*-acetyl amino acids are not involved [6—13], we have made an attempt to interpret the N-terminal acetylation as a post-initiation modification of polypeptide chains.

The transition of *Escherichia coli* ribosomal protein L_{12} to L_7 by acetylation of the N-terminal serine is a recent example of such a process [1]. In higher

organisms the problem of acetylation of *N*-α-amino acids is still unsettled probably with exception of the ε-acetylation of lysine residues in histones [14—16]. In the present paper we describe a cell-free system derived from eye lens tissue which provides an excellent tool to study the process of N-terminal acetylation. With the aid of this system it will be demonstrated that acetylation of eye lens proteins is not a part of the initiation mechanism.

EXPERIMENTAL PROCEDURE

Materials

L-[^{35}S]Methionine (spec. act. 20—40 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, England). Lens tRNA was isolated, purified and aminoacylated with [^{35}S]methionine as described previously [17]. The chemicals needed for the synthesis of the tetrapeptide Met-Asp-Ile-Ala were obtained from Bachem (Switzerland). Thermolysin and T_1 RNAase were products of Calbiochem.

T_1-RNAase Digestion

Digestion of tRNA charged with [^{35}S]methionine was carried out in a total volume of 5 µl containing 0.1 mM EDTA, 0.01 M sodium acetate pH 5.2 and 5 µg T_1 RNAase. The solution was incubated in a sealed capillary tube at 37 °C for 20 min. The incubation mixture was immediately spotted on Whatman 3 MM paper and electrophoresis was performed at pH 3.8 for 3 h at 35 V/cm in a Savant electrophoresis

tank cooled with Varsol. The dried paper was placed in close contact with Kodak X-ray film (type RHP Royal X-omat) and exposed for 24 h. Paper strips containing little radioactivity were cut into 1-cm pieces after electrophoresis, which were counted in a liquid scintillation spectrometer using a toluene-based scintillator.

Preparation and Fractionation of Ribosome-Bound Peptides

Incubation of the lens lysate supplemented with lens polysomes was carried out under conditions for amino acid incorporation with [^{35}S]Met-tRNA^{fMet} as the radioactive label, as described previously [2]. The total volume was 5 ml. After incubation at 30 °C for 8 min, cycloheximide was added to a final concentration of 0.2 mg/ml. The incubation mixture was chilled in ice and layered over 2 ml 1 M sucrose in 0.1 M Tris-HCl pH 7.4, 0.15 M KCl and 5 mM magnesium acetate. Centrifugation was performed at $200000 \times g$ for 2 h in a Ti-50 rotor of a Spinco preparative ultracentrifuge at 2 °C. The ribosome precipitate was suspended in 1 ml water, adjusted to pH 10—11 with NaOH and incubated at 30 °C for 3 h. This treatment causes a cleavage of amino acids and nascent peptides from tRNA in the ribosomal complex. The suspension was adjusted to pH 3 with formic acid and the insoluble material was removed by centrifugation. The solution containing ribosome-bound amino acids and peptides was placed on a Sephadex G-25 "superfine" column (70 × 1.2 cm), equilibrated with 0.5 % formic acid. 1-ml fractions were collected in an automatic fraction collector; 0.1 ml of each even fraction was used for measurement of radioactivity. Fractions were pooled as indicated in Fig. 4, lyophilized and subjected to thermolysin digestion. The Sephadex G-25 column was previously calibrated with α-crystallin, glucagon and valine.

CHEMICAL PREPARATION OF TETRAPEPTIDE Met-Asp-Ile-Ala
Specificity of Proteolytic Enzymes

In our experiments concerning the N-terminal end of α-crystallin, it was a prerequisite to find a proteolytic enzyme capable of splitting off specifically an N-terminal di, tri- or tetrapeptide, acetylated as well as unacetylated, from the completed or uncompleted peptide chains. Therefore it was necessary to synthesize the N-terminal unacetylated tetrapeptide of α-crystallin: Met-Asp-Ile-Ala. This tetrapeptide was synthesized from the components benzyloxycarbonyl-L-aspartic acid β-tert-butyl ester, the dipeptide L-isoleucine-L-alanine and N-tert-benzyloxycarbonyl-L-methionine-N-hydroxysuccinimide ester as will be described elsewhere (Strous, unpublished results).

Table 1. *Specificity of proteolytic enzymes*

Enzyme	Substrate	Product(s)
Pronase	Met-Asp-Ile-Ala	Met, Asp, Ile and Ala
Subtilisin		Met, Met-Asp, Met-Asp-Ile, Met-Asp-Ile-Ala
Thermolysin		Met-Asp
Pepsin		None
Chymotrypsin		None
Pronase	Ac-Met-Asp-Ile-Ala	Ac-Met-Asp
Subtilisin		None
Thermolysin		Ac-Met-Asp
Pepsin		None
Chymotrypsin		None

A part of the tetrapeptide was acetylated with acetyl-N-hydroxysuccinimide ester to give Ac-Met-Asp-Ile-Ala: With these two peptides we have tested several proteolytic enzymes concerning their specificity (Table 1).

It can be concluded that only thermolysin exhibits the specificity required for identification of the acetylated as well as the unacetylated N-terminal dipeptide of α-crystallin.

Thermolysin Digestion and Product Analysis

Thermolysin crystals were suspended on 0.2 M ammonium acetate buffer pH 8.5 and 5 mM CaCl$_2$ at a protein concentration of about 2 mg/ml; 0.1 M KOH was added until all the enzyme was dissolved and the pH was immediately adjusted to pH 8.5 with 0.1 M acetic acid. The enzyme solution was stored at −20 °C in small batches. Protein samples were dissolved in the same buffer (0.5—1.0 ml), thermolysin was added to a final concentration of 20—50 µg/ml and the digestion was performed at 37 °C for 4—6 h. After digestion the peptides were lyophilized, dissolved in distilled water and subjected to paper electrophoresis on Whatman 3-MM paper in acetic acid—pyridine—water, 6:200:794 (v/v/v) at pH 6.5 or 50:50:900 (v/v/v) at pH 4.5. In case unblocked peptides had to be removed the digested material was treated with Dowex-50 (H$^+$). For this treatment one volume of a Dowex suspension was added to the peptide solution, the mixture was shaken thoroughly, the resin was removed by centrifugation and the water layer containing the blocked peptides was lyophilized. Radioactivity on electropherograms was quantitated by cutting the paper into 1-cm strips which were counted in a liquid scintillation counter using a toluene-based scintillator. Reference peptides were stained for methionine using platinic iodine.
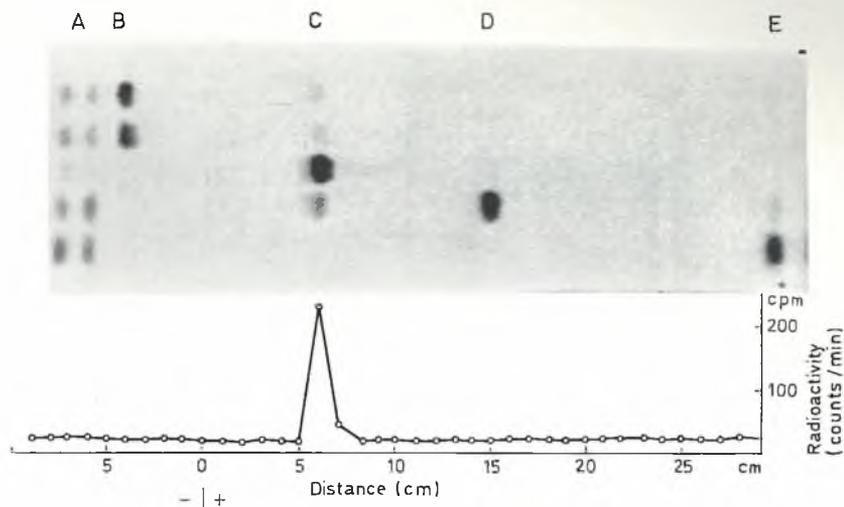
Fig. 1. *Electrophoresis of [³⁵S]methionine-labeled T₁ RNAase digests.* The following samples were subjected to high-voltage electrophoresis at pH 3.8 for 3 h at 40 V/cm. The lower part of the figure represents the RNAase T₁ digest of [³⁵S]Met-tRNA^fMet isolated from ribosomes after incubation in a crude lens cell-free system supplemented with lens polysomes in the presence of 140 μM sparcomycin [2]. The upper part of the figure is an autoradiogram of the following T₁ RNAase digests: (from top to bottom) [³⁵S]Met-tRNA₂^Met, [³⁵S]Met-tRNA₁^Met, [³⁵S]Met-tRNA^fMet and f[³⁵S]Met-tRNA^fMet; the lowest lane represents the radioactivity distribution after electrophoresis of the alkaline treated f[³⁵S]Met-tRNA^fMet. Zone (A) is free methionine and methionine-sulfoxide, zone (B) is the [³⁵S]methionyloligonucleotide derived from [³⁵S]-Met-tRNA^Met, zone (C) is the [³⁵S]Met-oligonucleotide derived from [³⁵S]Met-tRNA^fMet, zone (D) is the f[³⁵S]Met-oligonucleotide and Ac[³⁵S]Met-oligonucleotide derived from f[³⁵S]Met-tRNA^fMet and Ac[³⁵S]Met-tRNA^fMet (chemically acetylated) respectively and zone (E) is the position of formylmethionine (formylmethionine-sulfoxide is not visible)

RESULTS

Initiation of Protein Biosynthesis Inhibited by Sparsomycin

The presence of an acetyl group on the N-terminal methionine donated by Met-tRNA^fMet would be detected by examination of the products formed, when sparsomycin inhibits the growth of newly initiated chains. Therefore a crude lens cell-free system supplemented with additional lens polysomes was incubated under conditions for amino acid incorporation as described previously [2].

Sparsomycin was added at a concentration of 140 μM in order to inhibit peptide formation. [³⁵S]-Met-tRNA^fMet was used as radioactive label. After incubation for 10 min at 30 °C the ribosomes were isolated by centrifugation through a 1-M sucrose layer, the RNA was extracted from the pellet with phenol at pH 5.0, precipitated with ethanol and digested with T₁ RNAase. The products were subjected to paper electrophoresis at pH 3.8 (Fig. 1). All the radioactivity has the same mobility as the oligonucleotide product of the T₁ RNAase digestion of [³⁵S]Met-tRNA^fMet. No radioactivity could be detected at the position of the T₁ digestion product of Ac-Met-tRNA^fMet. Moreover since all the radioactivity bound to ribosomes could be detected in the water layer we may conclude that sparsomycin indeed inhibited formation of long peptides. In another series of experiments radioactivity transferred from [³⁵S]Met-tRNA^fMet into material bound to ribosomes was treated with trimethylamine and also subjected to paper electrophoresis at pH 6.5. It appeared that almost all ³⁵S label was present as methionine. Hence the conclusion is justified that dipeptide formation was inhibited almost completely at the sparsomycin concentration used.

When [³⁵S]Met-tRNA^Met was used under identical conditions as described for [³⁵S]Met-tRNA^fMet, the amount of radioactivity attached to the ribosomes was negligible indicating that the translation process was completely blocked.

These results demonstrate that the N-terminal acetylation of α-crystallin is not at the stage when initiation takes place.

State of Newly Formed Peptides

Once we have concluded that acetylation is not an integral part of protein initiation, the next step is to find an appropriate substrate in order to investigate the stage at which acetylation takes place. Our previous studies of the eye lens cell-free system [2] revealed that Met-tRNA^fMet starts the synthesis of α-crystallin and that the N-terminal methionine residue, carrying an acetyl group, is not removed during chain growth. To ascertain whether or not α-crystallin is the only water-soluble lens protein that can be labeled with [³⁵S]Met-tRNA^fMet we have incubated the cell-free system with [³⁵S]Met-tRNA^fMet
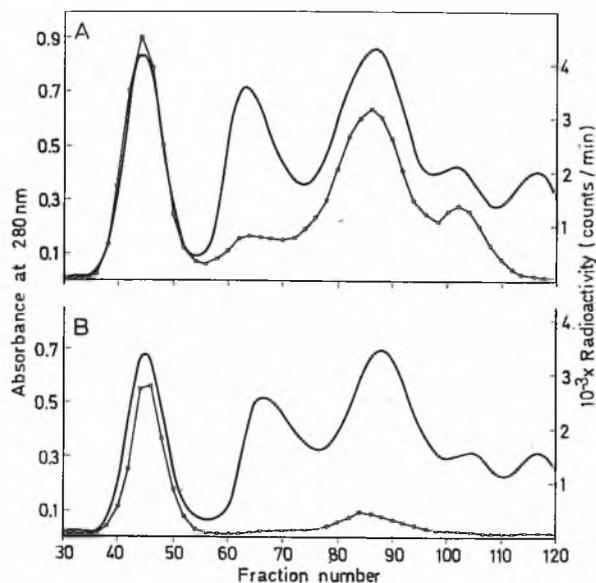
Fig. 2. *Gel filtration of a lens lysate on Sephadex G-200.* The incubation (final volume 2 ml) carried out as described previously [2], was passed through a Sephadex G-200 column (120 × 2 cm) equilibrated with 0.1 M Tris-HCl pH 7.8, 1 M NaCl and 0.1 mM EDTA. The effluent was directed through a 0.5-cm path-length flow cell mounted in a recording spectrophotometer set at 280 nm and then to an automatic fraction collector. Fractions of 4 ml were collected. To determine the radioactivity 0.1-ml samples were taken, the protein was precipitated with 5% trichloroacetic acid, filtered on glass fiber filters and the radioactivity was counted in a liquid scintillation counter using a toluene-based scintillator. (A) Incubation mixture labeled with [35S]methionine; (B) incubation mixture labeled with [35S]Met-tRNA[fMet]. The elution profile of absorbance at 280 nm reflects the separation of four classes of crystallins according to molecular weight α, β-high, β-low and γ-crystallin (——) absorbance 280 nm; (O–O–O) radioactivity

for 1 h at 30 °C. In order to determine which protein has been labeled, the incubation mixture, after alkaline treatment and dialysis to remove unreacted [35S]methionine, was subjected to chromatography on Sephadex G-200 under conditions described previously [19]. The gel-filtration step separates four classes of eye lens crystallins according to their molecular weight, in α, β_H, β_L and γ-crystallins. It appears (Fig. 2B) that the methionine donated by the initiator tRNA is incorporated almost exclusively into the α-crystallin molecule. A very small amount of radioactivity can be detected in the β_L region, indicating that still another newly synthesized polypeptide chain carries an N-terminal methionine residue. Fig. 2A shows the radioactivity pattern, if instead of [35S]Met-tRNA[fMet], [35S]methionine is used. It can be seen that in the latter case all the four classes of crystallins have been synthesized to a certain degree. Thermolysin appeared to be a useful tool for the determination of the N-terminus of
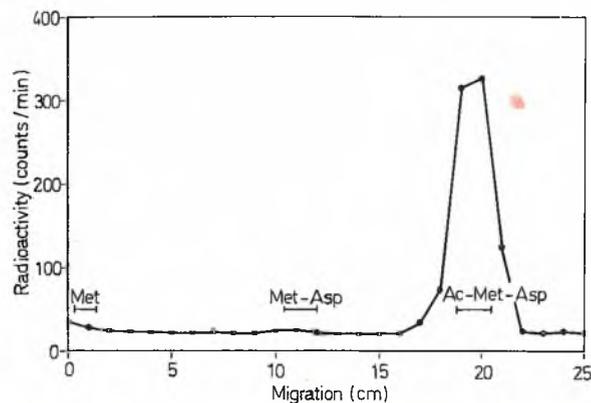

Fig. 3. *Identification of the N-terminal-peptide of α-crystallin, purified on Sephadex G-200 (compare Fig. 2B).* [35S]Met-tRNA[fMet]-labeled α-crystallin was digested with thermolysin and analyzed by high-voltage paper electrophoresis at pH 6.5 as described in the Method section

α-crystallin as it cleaves the N-terminal dipeptide Met-Asp in the unacetylated as well in the acetylated state. Analysis of the thermolytic digestion products of α-crystallin which has been labeled with [35S]Met-tRMA[fMet] and was purified on Sephadex G-200 revealed that all N-terminal methionine residues are acetylated. No radioactivity could be detected in the Met-Asp region of the electropherogram (Fig. 3).

It is known from association experiments that the polypeptide chains of α-crystallin having a molecular weight of about 20000 [20] reaggregate quickly and quantitatively into large complexes of molecular weight about 8×10^5 [21]. Since all polypeptide chains composing the α-crystallin aggregate are acetylated in the N-terminal position and no unacetylated α-chains have ever been found, the conclusion is justified that acetylation must have occurred before incorporation of the newly formed chains into the high-molecular-weight α-crystallin aggregate.

N-Terminal Acetylation, a Ribosomal Event

In order to study a possible acetylation during chain elongation, ribosome-bound peptides were examined for the presence of an N-terminal acetyl group. The cell-free extract was incubated under standard conditions with [35S]Met-tRNA[fMet]. After 8 min of incubation at 30 °C the reaction mixture was chilled, cycloheximide was added to a final concentration of 0.2 mg/ml and the ribosomes were isolated by centrifugation through a layer of sucrose. The ribosome-bound peptides were dissociated from the ribosomes as described in the Method section. The nascent chains were fractionated according to their length on a Sephadex G-25 "superfine" column. The elution profile of the peptides is shown in Fig. 4.
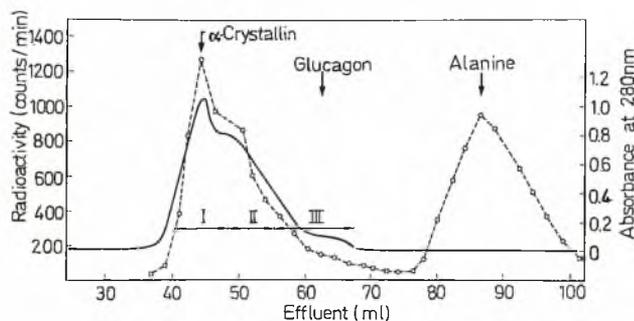
Fig. 4. *Elution profile of ribosome-bound peptides from a Sephadex G-25 column.* Peptides and amino acids isolated from ribosomes were solubilized by alkaline treatment, placed on the column and eluted with 0.5% formic acid; 0.1-ml fractions were used for measurement of radioactivity. (———) Absorbance at 280 nm; (O————O) radioactivity. Radioactivity was derived from [^{35}S]Met-tRNA^{fMet}

Fraction I consists of completed or nearly completed chains, fraction II contains peptides larger than glucagon and fraction III should consist of peptides with a molecular weight of about 3000.

Radioactivity eluted behind fraction III is mainly due to free methionine and some other unidentified components. The three fractions were lyophilized and the material was digested with thermolysin. To the supernatant, containing completed polypeptides RNAase was added to destroy the remaining [^{35}S]Met-tRNA and the solution was dialyzed against water and also treated with thermolysin. The thermolysin digests were analyzed by high-voltage paper electrophoresis at pH 6.5. Results of the analysis are shown in Fig. 5. In Fig. 5 A the distribution of radioactivity after digestion of the completed chains is depicted. It appears that all peptides are acetylated at that stage. Fig. 5 B, C and D represent the radioactivity patterns of fractions I, II and III, respectively. It can be seen that going from larger to smaller nascent peptides the amount of radioactivity in the Ac-Met-Asp region decreases as compared to the amount of radioactivity in the region of Met-Asp. In Fig. 5 B the ratio Ac-Met-Asp to Met-Asp is about 3 : 1; in Fig. 5 D this ratio is about 1 : 1. The radioactivity migrating between Ac-Met-Asp and Met-Asp is due to degradation of Ac-Met-Asp to Ac-Met and the radioactivity in the position of methionine in Fig. 5 B, C and D is probably a result of degradation of Met-Asp.

From Fig. 2 B it can be seen that a relatively low amount of radioactivity due to N-terminal methionine is also present in the β_L region. Enzymic studies revealed that this radioactivity does not contribute to the radioactivity found in the Met-Asp and Ac-Met-Asp region originating from α-crystallin chains (unpublished results).
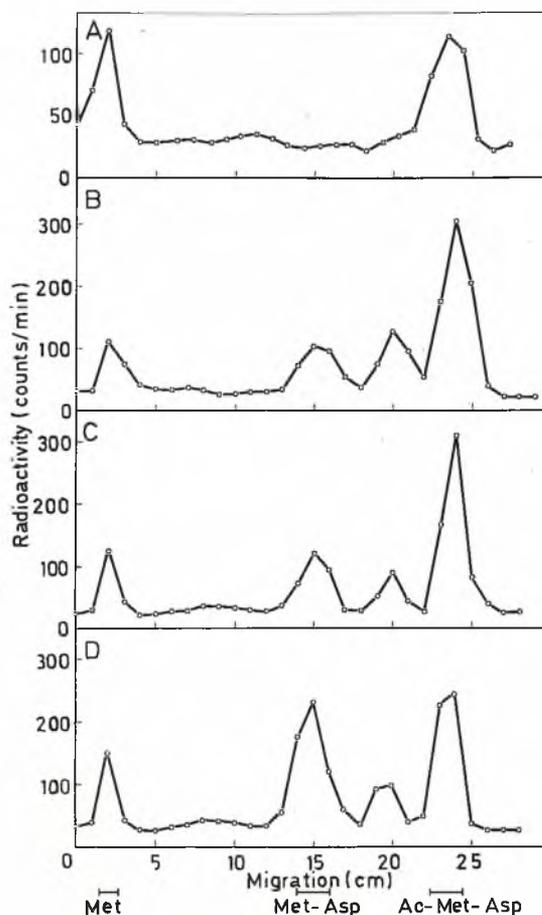

Fig. 5. *Electrophoretic analysis of the N-terminal dipeptides of α-crystallin after digestion with thermolysin.* A .5-ml reaction mixture containing [^{35}S]Met-tRNA^{fMet} was incubated at 30 °C for 8 min as described previously [2]. Completed chains were separated from nascent peptides; the nascent chains were fractionated according to their molecular weight (Fig. 4). Each fraction was digested with thermolysin and analyzed by high-voltage paper electrophoresis at pH 6.5 for 2 h at 50 volts/cm. 1-cm strips were cut out and the radioactivity was counted in a liquid scintillation counter. (A) Distribution of radioactivity after digestion of completed polypeptides; (B), (C) and (D) distribution of radioactivity after digestion of the fractions I, II and III from the Sephadex G-25 column (Fig. 4)

To ascertain the identity of the different components the products of the thermolytic digestion of fraction II were also treated with Dowex-50 (H$^+$) resin; this causes a disappearance of the radioactivity in the methionine and Met-Asp region (Fig. 6 A). Fig. 6 B shows the radioactivity pattern of the digestion of fraction II when electrophoresed at pH 4.5; the results appear to be completely in agreement with the electrophoretic analysis at pH 6.5.

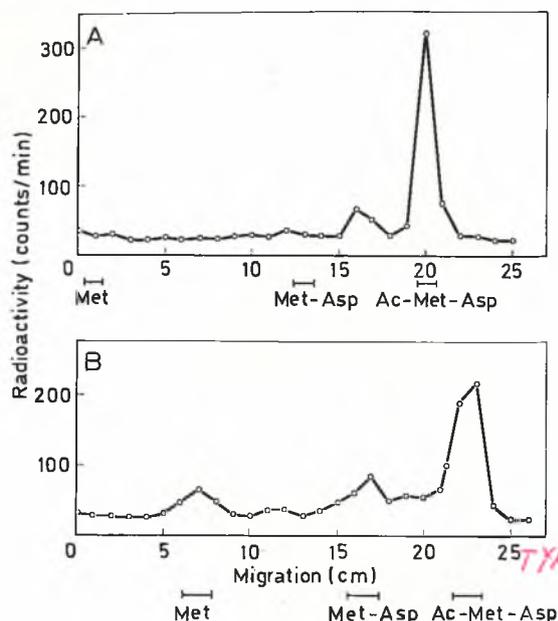These results are in favor of N-terminal acetylation during chain elongation.

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Fig. 6. *Identification of thermolytic peptides of fraction II in Fig. 5 C.* (A) Analysis of the radioactivity after treatment with Dowex-50 (H⁺); (B) re-electrophoresis of fraction II material at pH 4.5

Table 2. *N-Terminal sequence of acetylated proteins*

Protein	N-Terminal sequence	Reference
1. Hemoglobin fetal F_1 (human)	Ac-Gly-His-Phe	[23]
2. Ovalbumin	Ac-Gly-Ser-Gly	[24]
3. Cytochrome *c* (chicken)	Ac-Gly-Asp-Ile	[25]
4. Cytochrome *c* (human)	Ac-Gly-Asp-Val	[26]
5. Haemoglobin (carp)	Ac-Ser-Leu-Ser	[27]
6. Histone F2a2	Ac-Ser-Gly-Arg	[28]
7. Myosin (rabbit muscle)	Ac-Ser-Ser-Asp	[29]
8. Apoferritin (horse spleen)	Ac-Ser-Ser-Gln	[30]
9. Melanocyte-stimulating hormone (pig)	Ac-Ser-Tyr-Ser	[31]
10. Tabacco mosaic virus coat protein	Ac-Ser-Tyr-Ser	[32]
11. Cytochome *c* (wheat)	Ac-Ala-Ser-Phe	[33]
12. Enolase (rabbit muscle)	Ac-Ala-Gly-Lys	[34]
13. Fibrinopeptide (bovine)	Ac-Thr-Glu-Phe	[35]
14. Lactate dehydrogenase (dogfish)	Ac-Thr-Ala-Leu	[36]
15. α-Crystallin (bovine)	Ac-Met-Asp-Ile	[37]
16. Turnip yellow mosaic virus coat protein	Ac-Met-Glu-Ile	[38]
17. Tropomyosin (rabbit muscle)	Ac-Met-Asp-Ile	[39]

DISCUSSION

The results show that acetylation of the N-terminus of α-crystallin takes place after initiation and before completion of the chains. In our experiments advantage was taken from the fact that in spite of the use of a very crude cell-free system (S15), it was possible to label specifically the N-terminal amino acid of the α-crystallin polypeptides.

From our data one may also conclude that, of the proteins made in the cell-free system besides α-crystallin, only one other minor component in the β-low region of the Sephadex G-200 column bears a methionine residue in the N-terminal position. It is known from studies on the structure of the eye lens crystallins that three of the four classes are acetylated N-terminally namely α-, β_H- and β_L-crystallin while the γ-crystallins have a free N-terminal amino acid. Taking these facts into consideration one may argue that N-terminal acetylation is not dependent on a single specific amino acid (*i.e.* methionine). Apparently a more complicated signal, *e.g.* a certain amino acid sequence or a secondary structure, is required.

Our results further indicate that the acetylation of α-crystallin takes place on the ribosome. This does not necessarily exclude the occurrence of a soluble enzyme catalyzing the acetylation and/or acetylation in the absence of ribosomes.

Another feature is also noteworthy: α-crystallin is a rather unique eukaryotic protein in that its N-terminal methionine residue, donated by the initiator tRNA and which becomes acetylated during peptide growth, is not removed from the polypeptide chain. At the moment at least four different amino acids other than methionine, are known which are N-terminally acetylated (Table 2). Assuming that the same initiation mechanism with Met-tRNA^{fMet} is operative in the synthesis of all proteins on 80-S ribosomes, it seems that removal of the N-terminal methionine residue by a special ribosome-bound aminopeptidase generally occurs before the growing peptide chain becomes accessible to acetylation. This lends support to the idea that in the synthesis of α-crystallin, acetylation of the N-terminal methionine is not related to the fact that it remains attached to the chain. In other words the requirements for the removal of the methionine residue from the polypeptide chains and for N-terminal acetylation are not identical. Cleavage of the methionine residue may be prevented by the nature of the adjacent amino acid residue(s) which might determine the specificity of the splitting enzyme. In this connection it should be mentioned that in *E. coli* a ribosome-bound aminopeptidase cleaves all methionine dipeptides tested with the exception of Met-Asp [23]. The only other hitherto known N-terminal acetylated methionine residue occurs in the coat protein of turnip yellow mosaic virus particles and in tropomyosin from rabbit muscle. It is remarkable that the corresponding N-terminal tripeptides are quite similar to Ac-Met-Asp-Ile derived from α-crystallin.

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