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Synthesis of Lens Protein \textit{in vitro} \\ N-Terminal Acetylation of \(\alpha\)-Crystallin

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N-terminal acetylation of the lens protein \(\alpha\)-crystallin has been studied in a lens cell-free system. This system is capable of synthesizing \textit{de novo} \(\alpha\)-crystallin, a protein with acetylmethionine as N-terminus. When \([^{35}\text{S}]\)Met-tRNA\text{Met} is used as radioactive precursor only the \(\alpha\)-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 \textit{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-\(\alpha\)-amino acids is still unsettled probably with exception of the \(\varepsilon\)-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.

	extbf{EXPERIMENTAL PROCEDURE}

	extbf{Materials}

\(L-[^{38}\text{S}]\)Methionine (spec. act. 20—40 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, England). Lens tRNA was isolated, purified and aminoacylated with \([^{38}\text{S}]\)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.

\textbf{T\(_1\)-RNAase Digestion}

Digestion of tRNA charged with \([^{38}\text{S}]\)methionine was carried out in a total volume of 5 \(\mu\)l containing 0.1 mM EDTA, 0.01 M sodium acetate pH 5.2 and 5 \(\mu\)g T\(_1\) RNAase. The solution was incubated in a sealed capillary tube at 37 \(\degree\)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
N-Terminal Acetylation of α-Crystallin


peptide of α-crystallin: Met-Asp-Ile-Ala. This tetrapeptide was synthesized from the completed or unacetylated peptide chains. Therefore it was necessary to synthesize the N-terminal unacylated tetrapeptide of α-crystallin: Met-Asp-Ile-Ala. This tetrapeptide was synthesized from the components benzyl-oxycarbonyl-L-aspartic acid β-tert-butyl ester, the dipeptide L-isoleucine-L-alanine and N-tert-benzyl-oxycarbonyl-L-methionine-N-hydroxysuccinimide ester as will be described elsewhere (Strous, unpublished results).

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 [35S]Met-tRNA Met as the radioactive label, as described previously [2]. The total volume was 5 ml. After incubation at 30 °C for 5 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 × g for 2 h in a Ti-50 rotor of a Spincce 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 unacylated, from the completed or uncompleted peptide chains. Therefore it was necessary to synthesize the N-terminal unacylated tetrapeptide of α-crystallin: Met-Asp-Ile-Ala. This tetrapeptide was synthesized from the components benzyl-oxycarbonyl-L-aspartic acid β-tert-butyl ester, the dipeptide L-isoleucine-L-alanine and N-tert-benzyl-oxycarbonyl-L-methionine-N-hydroxysuccinimide ester as will be described elsewhere (Strous, unpublished results).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Product(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pronase</td>
<td>Met-Asp-Ile-Ala</td>
<td>Met, Met-Asp,</td>
</tr>
<tr>
<td></td>
<td>Met, Met-Asp, Met-Asp-Ile-Ala</td>
<td>Met-Asp-Ile-Ala</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>Met-Asp</td>
<td>None</td>
</tr>
<tr>
<td>Thermolysin</td>
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<td>None</td>
</tr>
<tr>
<td>Pepsin</td>
<td>Ac-Met-Asp-Ile-Ala</td>
<td>Ac-Met-Asp</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>Ac-Met-Asp-Ile-Ala</td>
<td>Ac-Met-Asp</td>
</tr>
</tbody>
</table>

Thermolysin digestion and Product Analysis

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 specificitiy (Table 1).

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

Thermolysin crystals were suspended on 0.2 M ammonium acetate buffer pH 8.5 and 5 mM CaCl₂ 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 $[^{35}S]$methionine-labeled $T_1$ RNAase digestes. 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 $T_1$ digest of $[^{35}S]$Met-tRNA$^{Met}$ isolated from ribosomes after incubation in a crude lens cell-free system supplemented with lens polyosomes in the presence of 140 μM sparsomycin [2]. The upper part of the figure is an autoradiogram of the following $T_1$ RNAase digestes: (from top to bottom) $[^{35}S]$Met-tRNA$^{Met}$, $[^{35}S]$Met-tRNA$^{Met}$, $[^{35}S]$Met-tRNA$^{Met}$, $[^{35}S]$Met-tRNA$^{Met}$ and $[^{35}S]$Met-tRNA$^{Met}$; the lowest lane represents the radioactivity distribution after electrophoresis of the alkaline treated $[^{35}S]$Met-tRNA$^{Met}$. Zone (A) is free methionine and methionine-sulfoxide, zone (B) is the $[^{35}S]$methionyloligonucleotide derived from $[^{35}S]$Met-tRNA$^{Met}$, zone (C) is the $[^{35}S]$Met-oligonucleotide derived from $[^{35}S]$Met-tRNA$^{Met}$, zone (D) is the $[^{35}S]$Met-oligonucleotide and Ac$[^{35}S]$Met-tRNA$^{Met}$ and Ac$[^{35}S]$Met-tRNA$^{Met}$ (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$^{Met}$ 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 polyosomes 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 peptidase formation. $[^{35}S]$Met-tRNA$^{Met}$ 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 $T_1$ 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 $T_1$ RNAase digestion of $[^{35}S]$Met-tRNA$^{Met}$. No radioactivity could be detected at the position of the $T_1$ digestion product of Ac-Met-tRNA$^{Met}$. 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 $[^{35}S]$Met-tRNA$^{Met}$ into material bound to ribosomes was treated with trimethylamine and also subjected to paper electrophoresis at pH 6.5. It appeared that almost all $^{35}S$ label was present as methionine. Hence the conclusion is justified that dipeptide formation was inhibited almost completely at the sparsomycin concentration used.

When $[^{35}S]$Met-tRNA$^{Met}$ was used under identical conditions as described for $[^{35}S]$Met-tRNA$^{Met}$, 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$^{Met}$ 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 $[^{35}S]$Met-tRNA$^{Met}$ we have incubated the cell-free system with $[^{35}S]$Met-tRNA$^{Met}$.
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 x 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-tRNAfMet. The elution profile of absorbance at 280 nm reflects the separation of four classes of crystallins according to molecular weight, α, β-hi, 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, βHI, βL 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 βL 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-tRNAfMet, [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 α-crystallin as it cleaves the N-terminal dipeptide Met-Asp in the unacylated as well as the acetylated state. Analysis of the thermolytic digestion products of α-crystallin which has been labeled with [35S]Met-tRNAfMet 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 x 10^6 [21]. Since all polypeptide chains composing the α-crystallin aggregate are acetylated in the N-terminal position and no unacylated 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-tRNAfMet. 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.
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 polypep-
tides RNAase was added to destroy the remaining 
[35S]Met-tRNA and the solution was dialyzed against 
water and also treated with thermolysin. The thermo-
lysin digests were analyzed by high-voltage paper 
electrophoresis at pH 6.5. Results of the analysis are 
shown in Fig.5. In Fig.5A the distribution of radio-
activity after digestion of the completed chains is 
depicted. It appears that all peptides are acetylated 
at that stage. Fig.5B, C and D represent the radio-
activity 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 region of Met-Asp 
decreases as compared to the amount of radioactivity in the region of Met-Asp. In Fig.5B the ratio Ac-Met-Asp to Met-Asp is about 3:1; in 
Fig.5D 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.5B, 
C and D is probably a result of degradation of Met-
Asp.

From Fig.2B it can be seen that a relatively low 
amount of radioactivity due to N-terminal methio-
nine is also present in the β1 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).

To ascertain the identity of the different compo-
ents 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.6A). 
Fig.6B shows the radioactivity pattern of the diges-
tion 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 acetyla-
tion during chain elongation.
**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- and γ-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<sup>Met</sup> 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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REFERENCES

18. Reference deleted.

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