N-TERMINAL ACETYLATION OF THE NASCENT CHAINS OF α-CRYSTALLIN

G.J.A.M. Strous, A.J.M. Berns and H. Bloemendal*

Department of Biochemistry

University of Nijmegen, Nijmegen, The Netherlands

Received April 18, 1974

SUMMARY

Labeling of a cell-free extract derived from eye lens tissue with (^{35}S)Met-tRNA^{fMet} results in α-crystallin polypeptides which bear acetyl-(^{35}S)methionine in N-terminal position. An attempt has been made to determine the exact moment of acetylation. For that purpose peptidyl-tRNAs were isolated in a one-step procedure which is based on a specially designed sucrose gradient referred to as strip-gradient. Separation of the nascent chains has been carried out according to their molecular weights. Our previous suggestion that N-terminal acetylation takes place while the peptide is still on the ribosome was confirmed. It is demonstrated that acetylation starts when the polypeptide chain is about 25 amino acids long and is virtually completed when the chains have reached a length of about 50 amino acids. Acetyl-CoA appears to deliver the acetyl group for the N-terminal acetylation.

INTRODUCTION

N-terminal acetylation of prokaryotic as well as eukaryotic proteins has been proved to occur after peptide chain initiation (1, 2, 3). Until now not only the mechanism of N-terminal acetylation is unclear but also its significance is poorly understood. With regard to the moment of acetylation some data are available. The prokaryotic ribosomal protein L_{12} can be acetylated enzymatically after completion (1). In eukaryotic proteins N-terminal acetylation has been suggested to be a post-initiational rather than a post-translational modification (2, 3). In this paper we deal with the N-terminal acetylation of the polypeptides of α-crystallin, a major structural eye lens protein. A cell-free system derived from calf eye lens tissue, which is capable to initiate new polypeptides, has been used in order to obtain labeled nascent chains. If the labeling is performed with (^{35}S)Met-tRNA^{fMet} only α-crystallin polypeptides

* to whom requests for reprints should be addressed

can be labeled N-terminally in contrast to the polypeptides of β- and γ-crystallin (2). The explanation is found in that α-crystallin has an acetylmethionine residue in N-terminal position (4). This feature offers the possibility to examine the very moment of acetylation during the synthesis of the α-crystallin polypeptides.

In order to isolate the nascent peptide chains we have developed a one-step procedure for the purification of peptidyl-tRNAs from the ribosomes. This method allows a high purification of the ribosome-bound peptidyl-tRNAs in a yield of almost 100 percent.

The availability of the lens cell-free system together with the new method for purification of the nascent polypeptides enables to answer the question when and how N-terminal acetylation takes place.

MATERIALS AND METHODS

(^{3}H)Acetyl-CoA (spec. act. 900 mCi/mmol) was obtained from New England Nuclear. Lens tRNA was isolated, purified and amino acylated with (^{35}S)methionine as described previously (5). The procedures for preparation of the lens lysate and for the in vitro synthesis of eye lens proteins have been reported elsewhere (6, 7).

For the isolation of peptidyl-tRNAs special gradients designated as strip gradients were prepared in tubes of the SB-283 rotor of an IEC. The lower layer (5.5 ml) contained 0.05 M Tris-HCl, pH 7.4, 0.08 M KCl and 0.01 M EDTA in a 20-30% (w/v) linear sucrose gradient. The upper layer (6.0 ml) contained 0.05 M Tris-HCl, pH 7.4, 0.08 M KCl, 0.005 M magnesium acetate and 0.005 M 2-mercaptoethanol in a 10-20% (w/v) linear sucrose gradient. The ribosomes carrying labeled peptidyl-tRNAs were isolated from the incubation mixture by pelleting at 150,000 g for two hours at 2° C in a Ti-50 rotor of a Spinco ultracentrifuge. The ribosomes were resuspended in 0.5 ml of a medium containing 0.05 M Tris-HCl, pH 7.4, 0.08 M KCl, 0.005 M magnesium acetate and 0.005 M 2-mercaptoethanol

and applied onto the strip gradient. Centrifugation was performed at 40,000 rpm for 6 hours at 2^{0} C in an IEC B-60 ultracentrifuge. The sucrose gradient was displaced by a heavy sucrose solution which was pumped under the gradient through a needle. The optical density was monitored with the aid of an LKB optical unit, coupled to a logarithmic recorder. Fractions of 0.9 ml were collected. The peptidyl-tRNAs were precipitated by adding 0.1 volume of 2 M potassium acetate, pH 5.0 and 2.5 volumes of cold ethanol. After 16 hours at -20^{0} C the precipitate was collected by centrifugation. The pellet was dissolved in 0.5 ml of 0.1 M NaOH and incubated at 37^{0} C for 15 min. The solution was acidified with formic acid and applied onto a Sephadex G-25 "fine" column (100 x 1.5 cm). The column was eluted with 0.5 M formic acid and the eluate was fractionated as indicated in fig. 2. The peptidyl-fractions were digested with thermolysin and analyzed for the presence of N-terminal acetyl-groups by high voltage paper electrophoresis at pH 6.5 (2).

RESULTS AND DISCUSSION

Isolation of nascent chains.

As described in the method section a strip-gradient consists of a linear sucrose gradient whose upper phase contains Mg^{2+} ions whereas the lower phase is supplemented with EDTA. Polysomes carrying peptidyl-tRNA which are loaded on the gradient will dissociate into their subunits when reaching the EDTA containing zone. In the same region the peptidyl-tRNAs will be stripped from the ribosomes. While the subunits sediment towards the bottom, the peptidyl-tRNAs virtually remain at the top of the EDTA layer. The radioactivity pattern in fig. 1. illustrates the isolation of peptidyl-tRNAs from a cell-free extract, derived from eye lens tissue which has been incubated with (^{35}S)Met-tRNA^{fMet}. The labeled material has the following properties:

(a) On a DEAE-Sephadex column it behaves as peptidyl-tRNA. It binds to the column and can be eluted with 0.4 M sodium chloride; (b) it can be quantitatively precipitated with 70% ethanol.

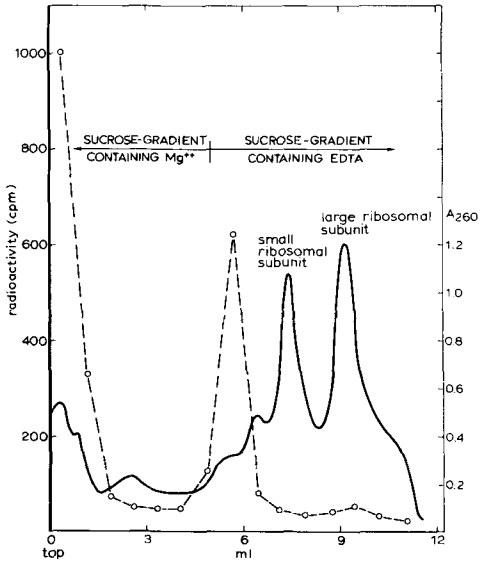

Fig. 1. Sedimentation profile of (^{35}S)methionine labeled calf lens ribosomes after strip gradient centrifugation. The lens cell-free extract was incubated with (^{35}S)Met-tRNA^{fMet} for 5 min at 37° C in a total volume of 5 ml as described previously (2). The ribosomes were pelleted and applied onto a strip-gradient as described in the method section. Aliquots of 0.05 ml were used to determine the radioactivity.

With the aid of the isolation procedure described rather pure preparations in a high yield are obtained. Only some ribosomal proteins released from the ribosomes by EDTA are present in the peptidyl-tRNA fraction. However, these contaminants do not interfere with the procedure.

The moment of N-terminal acetylation.

In order to determine the moment of N-terminal acetylation of α-crystallin polypeptides the nascent peptides, isolated as described above, have been separated according to their length on a Sephadex G-25 column. We have chosen this type of columns as previous studies showed that acetylation most probably occurs during an early stage of chain elongation (2). Separation of the nascent chains on the Sephadex G-25 column offers the possibility to examine peptides with a molecular weight between 1000 and 5000 dalton. The elution profile of

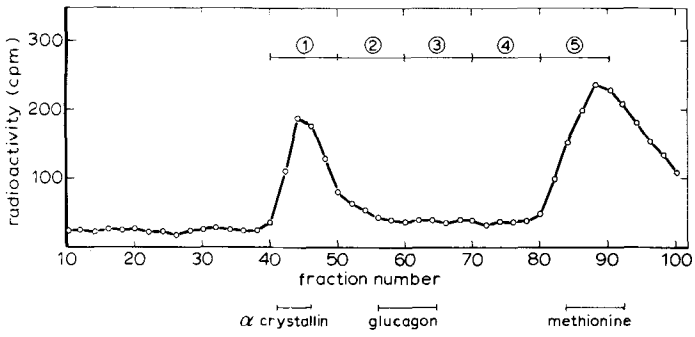

Fig. 2. Elution profile of (^{35}S)methionine labeled nascent lens crystallin chains from a Sephadex G-25 column.
The peptides and amino acids, obtained after strip-gradient centrifugation (see fig. 1) were applied onto a Sephadex G-25 column. The column was eluted with 0.5% formic acid. 1.5 ml fractions were collected. For measurement of the radioactivity aliquots of 0.1 ml were used. The column was calibrated with α-crystallin, glucagon and methionine. Five fractions were pooled as indicated.

the radioactive peptides is shown in fig. 2. The column fractions have been pooled to yield five ranges of molecular weights (MW), namely: 1) MW higher than 4000; 2) 4000 - 3000; 3) 3000 - 2000; 4) 2000 - 1500; 5) lower than 1500. These five fractions have been analyzed for the presence of an N-terminal acetyl group. Fig. 3 shows the radioactivity patterns of the thermolytic digests of the individual fraction upon high voltage paper electrophoresis at pH 6.5. Thermolysin cleaves α-crystallin polypeptides in the acetylated as well in the unacetylated state to give the dipeptides acetyl-Met-Asp and Met-Asp, respectively (2). No radioactivity in the acetyl-Met-Asp region can be detected in panel 4) (fig. 3) while an increasing ratio between acetyl-Met-Asp and Met-Asp is found in panels 3), 2) and 1), respectively.

This observation provides evidence that the acetylation process becomes operative when the polypeptide chain has a size corresponding to 2500 daltons. Apparently also longer peptides can be acetylated. In panel 1) 20 percent of the α-crystallin peptides, corresponding to sizes higher than 4000 dalton, is still unacetylated.

In the panels 4) and 5) in addition to unacetylated Met-Asp, identified by

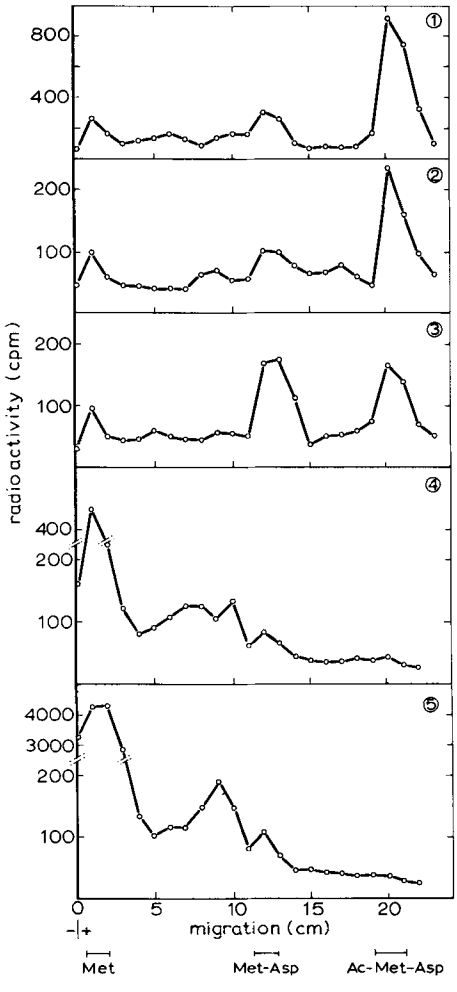

Fig. 3. Electrophoretic analysis of thermolytic digests of (^{35}S)methionine labeled nascent chains derived from α-crystallin.
The radioactive peptides isolated on strip-gradients and separated as indicated in fig. 2 were digested with thermolysin (2). The resulting peptides were subjected to high voltage paper electrophoresis at pH 6.5 in a water-cooled Savant electrophoresis apparatus at 50 V/cm for 2 hours. 1 cm strips were cut out and the radioactivity was counted in a liquid scintillation counter. The panels are numbered corresponding to the five fractions indicated in fig. 2. Reference peptides were stained for methionine using platinic iodide (8).

high voltage electrophoresis at different pH values, unidentified methionine peptides appear. Their occurrence can be explained as follows. Besides α-crystallin also β- and γ-crystallins are synthesized in the cell-free system (6). As the synthesis of all polypeptides starts with Met-tRNA^{fMet} the nascent

chains smaller than 2000 dalton will bear an N-terminal methionine residue. The radioactivity present in the unidentified peaks of the panels 4) and 5) may be due to the products of thermolytic digestion of the peptides derived from β- and γ-crystallin synthesizing polysomes labeled with (^{35}S)Met-tRNA^{fMet}. Radioactive material migrating slower than the dipeptide Met-Asp is only observed in panels4) and 5). This indicates that peptides, not related to α-crystallin chains, with a molecular weight up to 3000, contain still Met in N-terminal position, whereas in longer chains this Met is splitt of. So one may conclude that the aminopeptidase activity, which removes the N-terminal Met residue, becomes operative at about the same chain length as the enzyme which catalyzes the N-terminal acetylation of α-crystallin. This chain length (± 25 amino acid residues) corresponds to the size of the polypeptide chain which is supposed to be buried in the ribosome (9).

The data presented above give some information concerning the minimal length of the peptide required to become recognized by the acetylating enzyme. We have shown that growing peptide chains comprising about 20 amino acids can be acetylated. Therefore it seems to be plausible that only a rather small sequence of amino acids serves as recognition site for the acetylating enzyme. Since also rather long N-terminal polypeptides can be acetylated it suggests that the enzyme involved is not ribosomal bound. Whether the ribosome per se is involved in this process needs further investigation.

Acetylation mediated by acetyl-CoA.

In order to establish whether or not the N-terminal acetylation is accomplished with the aid of acetyl-CoA we have incubated the lens cell-free system with (^{3}H)acetyl-CoA. After an incubation time of 60 minutes α-crystallin has been isolated by gel filtration on a Sephadex G-200 column (2). Samples of the lyophilized protein have been digested with two enzymes as described previously. After pronase digestion of the native α-crystallin the N-terminal dipeptide Ac-Met-Asp is splitt off (10) whilst with subtilisin the N-terminal

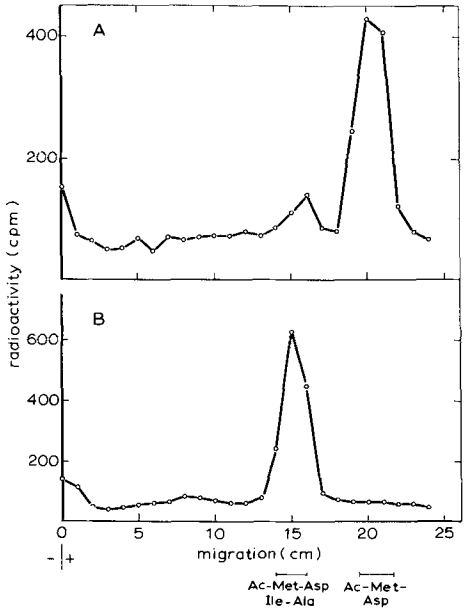

Fig. 4. Paper electrophoretic analysis of pronase (A) and subtilisin (B) digests from (^{3}H)acetyl labeled α-crystallin polypeptides.
In vitro protein biosynthesis was performed in a total volume of 1 ml for 60 min as described previously (6) except that 100 μCi of (^{3}H)acetyl-Coenzyme A was used as radioactive precursor. Labeled α-crystallin isolated by Sephadex G-200 gel filtration was digested and the resulting peptides were separated by high voltage paper electrophoresis at pH 6.5 as described in the legend to fig. 3. The paper strips were combusted with the aid of a Packard Tricarb sample oxidizer and the samples were counted with Insta-gel.

tetrapeptide Ac-Met-Asp-Ile-Ala can be recovered (11). The paper electrophoretic analysis of the ^{3}H-labeled digestion products is shown in fig. 4. It appears that the radioactivity can be ascribed to the N-terminal di- and tetrapeptide of α-crystallin, respectively. Pronase digestion also results in the appearance of a small amount of acetyl-methionine migrating just behind the acetylated dipeptide (compare fig. 4A).

The latter results provide evidence that acetyl coenzyme A serves as donor for the N-terminal acetyl group of α-crystallin.

ACKNOWLEDGEMENTS

The present investigations have partly been carried out under auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial

aid from the Netherlands Organization for the advancement of Pure Research (Z.W.O.).

REFERENCES

1. N. Brot and H. Weissbach, Biochem. Biophys. Res. Commun. 49, 673-679 (1972).
2. G.J.A.M. Strous, H. van Westreenen and H. Bloemendal, Eur. J. Biochem. 38, 79-85 (1973).
3. D.S. Shih and P. Kaesberg, Proc. Natl. Acad. Sci. U.S. 70, 1799-1803 (1973).
4. H.J. Hoenders and H. Bloemendal, Biochim. Biophys. Acta 147, 183-185 (1967).
5. G. Strous, J. van Westreenen and H. Bloemendal, FEBS Letters 19, 33-37 (1971).
6. G.J.A.M. Strous, H. van Westreenen, J. van der Logt and H. Bloemendal, Biochim. Biophys. Acta (1974) in the press.
7. G.J.A.M. Strous, A.J.M. Berns, H. van Westreenen and H. Bloemendal, Eur. J. Biochem. 30, 48-52 (1972).
8. C.W. Easley, Biochim. Biophys. Acta 107, 386-388 (1965).
9. R. Jackson and T. Hunter, Nature 227, 672-676 (1970).
10. H.J. Hoenders, J.J.G. Schoenmakers, J.J.T. Gerding, G.I. Tesser and H. Bloemendal, Exp. Eye Res. 7, 291-300 (1968).
11. H.J. Hoenders, J. van Tol and H. Bloemendal, Biochim. Biophys. Acta 160, 283-285 (1968).