The function of \( N^\text{\#} \)-acylation of the eye-lens crystallins

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The putative protective role of the \( N^\text{\#} \)-acyetyl group of proteins has been investigated. Synthetic, non-acylated \( N^\text{\#} \)-terminal tetrapeptides of the \( \alpha_2 \)- and \( \gamma \)-crystallin chains are good substrates for leucine aminopeptidase, while the acetylated ones are completely resistant. In the native, non-acylated, \( \gamma \)-crystallin the \( N \) terminus is not degraded by leucine aminopeptidase. Newly synthesized \( \alpha_2 \)-crystallin, in which the normally occurring \( N^\text{\#} \)-terminal acetylation has been prevented during cell-free translation, is virtually resistant against degradation by leucine aminopeptidase. Only at extreme enzyme-substrate ratios the \( N \)-terminal methionine is removed. Although the \( N^\text{\#} \)-acyetyl group by its very nature protects against this exopeptidase, we conclude that the group is not essential for this purpose in the native crystallins.

\( N^\text{\#} \)-terminal acylation is a widespread phenomenon in eukaryotes and viruses, and to a lesser extent in prokaryotes. Although virtually nothing is known about the underlying mechanism and functional implications of \( N^\text{\#} \)-acylation, the protection of proteins against proteolytic degradation by aminopeptidases has been suggested as a general function [1]. Experiments to support this hypothesis failed to show significant differences in the rate of turnover of acetylated and non-acylated forms of feline \( \beta \)-globin [2], and of proteins from mouse L-cells [3]. However, these cases have to be interpreted with great care, since the acetylated and non-acylated proteins were not of the same type. For the acetylated form of cytoplasmic actin from cultured Drosophila cells [4] it has been shown that the acetyl group protects against \( N \)-terminal degradation \textit{in vitro} by kidney leucine aminopeptidase. Until now, no decisive evidence has been presented for a protective role of \( N^\text{\#} \)-acylation \textit{in vivo}.

In bovine lens a relatively large amount of leucine aminopeptidase is present [5], while about 80% of the water-soluble calf lens proteins (the \( \alpha \) and \( \beta \)-crystallin chains) are \( N^\text{\#} \)-acylated [6]. Therefore, this system should be suitable for the investigation of a possible function of \( N^\text{\#} \)-acylation as protection against proteolysis. Here we describe the susceptibility of the \( \alpha \)-crystallin \( \alpha_2 \) chain and \( \gamma \)-crystallin, two of the major lens proteins [6], with respect to this enzyme.

\textbf{MATERIALS AND METHODS}

\textbf{Materials}

Oxaloacetate and citrate synthase were obtained from Sigma, Pico-Fluor 15 from Packard. Des-\( N^\text{\#} \)-Ac-\( \alpha_2 \)-A-(1-4)-tetrapeptide (\( \alpha_2 \)-Met-Asp-Ile-Ala), \( \alpha_2 \)-A-(1-4)-tetrapeptide (\( \alpha_2 \)-Met-Asp-Ile-Ala), \( \gamma \)-I-(1-4)-tetrapeptide (\( \gamma \)-Gly-Lys-Ile-Thr) and \( N^\text{\#} \)-Ac-I-(1-4)-tetrapeptide (\( N^\text{\#} \)-Ac-Gly-Lys-Ile-Thr) were synthesized by the classical approach. \( \gamma \)-Crystallin was isolated by gel filtration [6].

\textit{Enzymes.} \( N^\text{\#} \)-Acytransferase (EC 2.3.1.-); citrate synthase (EC 4.1.3.7); leucine aminopeptidase (EC 3.4.1.11); ribonuclease A (EC 3.1.27.5).

\textit{Fractionation of the lens}

Eyes from 3-6-month-old calves were obtained freshly from the slaughterhouse and kept on ice. For the determination of \( N^\text{\#} \)-acyltransferase activity lenses were removed and dissected into five fractions: (1) central region of the epithelial layer adhering to the capsule of the lens; (2) germinative region and the region of cellular elongation adhering to the capsule at the equator; (3) elongated fiber cells in the superficial cortical region (diameter, \( d \geq 12.0 \) mm); (4) fiber cells in the deeper layers surrounding the nucleus (\( d \geq 9.5-12.0 \) mm); (5) deep cortex and nucleus (\( d < 9.5 \) mm). To each of the fractions one volume of Sörensen's phosphate buffer, pH 7.4, was added, and the mixtures were homogenized at 0 °C. The homogenates were centrifuged at 15,000 × g for 10 min at 4 °C. The supernatant was used for determination of the enzymatic activity.

\( N^\text{\#} \)-Acytransferase assay

Details on the assay have been published elsewhere [7].

\textit{Isolation of calf lens \( \alpha_2 \)-mRNA}

Calf lens polyribosomes were isolated as described previously [8]. Poly(A)-rich RNA was selected using oligo(dT)-cellulose [9], \( \alpha_2 \)-mRNA was isolated by electrophoresis of poly(A)-rich RNA in a slab gel in the presence of methylmercury hydroxide [10].

\textit{Synthesis of lens proteins de novo}

Rabbit reticulocytes were prepared as described by Pelham and Jackson [11]. A 30,000 × g supernatant of the lysed cells was used as a cell-free system. The lysate was made mRNA-dependent by preincubation with micrococcal nuclease in the presence of CaCl\(_2\) for 15 min at 20 °C. Incubations were performed at 30 °C for 1.5 h under conditions as described previously [11]. \( \text{[\text{35}S]}\)Methionine was added as the only labeled amino acid.
In order to obtain newly synthesized, non-acetylated polypeptides, the endogenous acetylating capacity of the retinaldehyde lyase was suppressed by adding 2.4 μmol oxaloacetate and 72 units of citrate synthase/ml [12]. Purified αA2 mRNA was added at a concentration of 0.01 mg/ml. Translation was performed in 20-μl batches, and terminated by adding 0.2 mg of RNase A/ml and subsequent incubation at 37°C for 20 min.

**Leucine aminopeptidase digestion of polypeptides**

Leucine aminopeptidase was isolated from bovine lens and activated in the presence of Mn²⁺ ions [13]. The tetrapeptides and γ-crystallin were dissolved to final concentrations of 6–12 mg/ml in 0.1 M ammonium bicarbonate, pH 8.9, and activated leucine aminopeptidase was added in a 1–5% (w/w) enzyme/substrate ratio. Digestion was performed at 37°C. After different periods of time an aliquot was taken, and digestion was stopped by freezing in solid CO₂/acetone and lyophilization. Exposure of de novo synthesized chains to leucine aminopeptidase was performed as described for the peptides.

**Analysis of digests**

Analysis of de novo synthesized polypeptides after incubation with leucine aminopeptidase was performed by two-dimensional gel electrophoresis [14]. Radioactive spots were visualized by scintillation autoradiography. Protein spots were cut from the gel, solubilized in a mixture of ammonia and hydrogen peroxide (1:19, v/v) at 60°C, and counted using Pico-Fluor 15 as a scintillation agent. In the case of γ-crystallin, the amino acids possibly released were separated from undigested chains by gel filtration on a Bio-Gel P4 column (120 × 1 cm) in 1% ammonium bicarbonate at a flow rate of 2.2 ml/h. Relevant fractions were lyophilized and their amino acid contents analyzed. In the experiments with the N-terminal tetrapeptides of αA2 and γII-crystallin the digests were analyzed by peptide mapping [15]. Relevant spots were subjected to amino acid analysis.

**RESULTS AND DISCUSSION**

**Distribution of N⁰-acetyltransferase and leucine aminopeptidase in bovine lens**

In order to determine the various stages in lens differentiation where N⁰-acetylation and proteolytic degradation of crystallins may occur, we compared the activity of N⁰-acetyltransferase and leucine aminopeptidase in the different layers of the calf lens. The N⁰-acetyltransferase activity in the supernatant of each fraction was determined (Fig. 1). Fractions I and II each contain only 1% of the total enzyme activity, while fraction III, which comprises half the volume of the lens, contains 84% of the enzyme activity. Therefore, it is clear that the enzyme is mainly present in those regions where crystallin synthesis takes place [6]. This is in accordance with the fact that N⁰-acetylation of the γ-crystallin A2 chain occurs as soon as the nascent chain emerges from the ribosome [16].

On the other hand, the enzyme leucine aminopeptidase is found in all layers of the cortex and nucleus, but not in the epithelium [17]. The highest activity is present in those areas where protein synthetic activity ceases. Since the nucleus of the lens contains intact crystallins which are almost as old as the organ itself [6], these proteins would have to be protected against proteolytic attack by this enzyme during the lifespan. N⁰-Acetylation of the α- and β-crystallins might give this protection. A question that arises immediately, however, is why γ-crystallin is not acetylated at its N terminus.

**N-terminal tetrapeptides of γI-I and αA2-crystallin as substrates for leucine aminopeptidase**

The sensitivity to manganese-activated leucine aminopeptidase was established for both the acetylated and non-acetylated forms of the N-terminal sequences of α- and γ-crystallin. The acetylated αA2-(1–4)-tetrapeptide, which corresponds to the N-terminal sequence of the αA2 chain as it occurs in vivo, is completely resistant, as is the N⁰-Ac-γI-(1–4)-tetrapeptide, the N-terminal sequence of the γI chain which is non-acetylated in vivo. The non-acetylated tetrapeptides, however, are good substrates for leucine aminopeptidase. Using a 1% enzyme/substrate ratio (w/w), the N-terminal methionine of des-N⁰-Ac-αA2-(1–4)-tetrapeptide is lost within 1 min, quickly followed by aspartic acid in position 2 (Fig. 2A). The remaining dipeptide Ile-Ala is further degraded (data not shown). The degradation of non-acetylated γI-crystallin tetrapeptide by leucine aminopeptidase is less rapid. The N-terminal glycine is lost within several hours, while the next three amino acids are then liberated within seconds, as concluded from the fact that no tri- or dipeptides are observed (Fig. 2B).

**Exposure of γ-crystallin to leucine aminopeptidase**

When native γ-crystallin chains were exposed to leucine aminopeptidase no amino acids could be removed from the N terminus, even at an enzyme/substrate ratio (units/nmol) approximately 120-times higher than the one used for the tetrapeptides. It thus appears that the three-dimensional structure of γ-crystallin makes this protein completely resistant to leucine aminopeptidase. This is understandable, since the N-terminal sequence of γ-crystallin II chain is firmly embedded within the molecule [18].
**Synthesis of non-acetylated αA chains in a reticulocyte cell-free system**

In order to establish whether the Nα-acetyl group in native αA chain is required for protection against aminopeptidase degradation, we had to synthesize non-acetylated αA chains. This can be accomplished by adding oxaloacetate and citrate synthase to a reticulocyte cell-free system just before addition of mRNA. In this way the endogenous pool of acetyl-coenzyme A is metabolized in the citric acid cycle and Nα-acetylation of newly synthesized chains is inhibited [12].

We subjected total calf lens poly(A)-rich RNA to electrophoresis in a denaturing slab gel, and selected the αA2 mRNA fraction. Translation of this mRNA gives a more clear-cut picture on two-dimensional gel as compared with translation of total poly(A)-rich RNA.

Stained gel patterns of α-crystallin normally show four subunits, i.e. αB2, αB1, αA3, and αA1, where αA1 (pl 5.4/5.6) is the deamidated form of αA2 (pl 5.7/5.92) [19] (Fig. 3B). Translation of αA2 mRNA without prevention of acetylation directs the synthesis of αA2 as the main product. In addition a small amount of presumably deamidated product, which does not comigrate with αA1, can be seen (Fig. 3A and B). Under inhibiting conditions, the main product is in a more basic position (pl 5.9/6.1) as indeed can be expected for the non-acetylated αA chain (Fig. 3C). Inhibition of acetylation apparently is not complete, because a minor amount of the acetylated αA2 chain is still present. We suppose that this is caused by the addition of mRNA to the translation mixture almost immediately after oxaloacetate and citrate synthase have been added. The relative amounts of acetylated and non-acetylated αA chains can conveniently be estimated by determination of the amount of radioactivity in the protein spots from the gels, since no significant overlap between the two spots occurs.

**Exposure of newly synthesized αA2 and des-Nα1-Ac-αA to leucine aminopeptidase**

When des-Nα1-Ac-αA is exposed to leucine aminopeptidase the following degradation steps may be expected. Initially, the N-terminal 35S-labeled methionine may be removed, resulting in a 50% decrease in radioactivity of the polypeptide, if the only methionine is present at position 138 [6]. No shift in position on two-dimensional gels would result after this step. Further degradation would then remove aspartic acid in position 2, resulting in a change of isoelectric point of the shortened polypeptide. On the gel this would give rise to a shift towards the cathode. A mixture of acetylated and non-acetylated αA (ratio 0.84) was exposed to leucine aminopeptidase at an enzyme-substrate ratio of 0.05 unit/nmol, or about 10-times higher than that used for the tetrapeptides. After 1 h no shift on two-dimensional gels was seen, nor was there a decrease in the ratio between the two αA chains (Fig. 4A). Only at an extreme enzyme-substrate ratio (0.851 unit/nmol, which is about 200-times higher than for the tetrapeptides) was a rapid decrease in the ratio des-Nα1-Ac-αA/αA2 observed within 1 h (Fig. 4B) without a concomitant shift of the gel pattern, indicating the loss of the N-terminal methionine. It thus may be concluded that the non-acetylated form of αA is well protected against proteolytic degradation by leucine aminopeptidase. Only extreme and unphysiological amounts of enzyme are able to remove the N-terminal methionine.

In the interpretation of these experiments one has to keep in mind that αA synthesized under the direction of αA2 mRNA in a reticulocyte lysate remains in the monomeric form [20]. This is in contrast to the situation in vivo, where αA2 is found together with αB2 in complexes with an average relative molecular mass of 800 000 [6]. It is very likely that this aggregation makes the αA chains in vivo even more resistant to proteolytic breakdown.

In our experiments lenticular leucine aminopeptidase has been activated with manganese ions; *in vivo* this enzyme occurs in a less active form [21], while glutathione, an inhibitor of the enzyme is also present in considerable amounts in the lens [22]. Therefore, it is not easily apparent to what extent leucine aminopeptidase exhibits its proteolytic action *in vivo*.

We have shown here that γ-crystallin and non-acetylated αA-crystallin are resistant to proteolytic degradation by leucine.
aminopeptidase in vitro, although their N-terminal sequences are good substrates for this enzyme. We conclude that the three-dimensional structure of both proteins is a stabilizing feature. The experiments described in this paper thus do not support the idea that the function of N\(^2\)-acetylation, at least in α-crystallin, is protection against proteolysis of native protein. It has, however, to be kept in mind that the only moment when \(\alpha\)A is not protected by its three-dimensional structure may be during its synthesis, when the nascent chain emerges from the ribosome. The N\(^2\)-acyl group, which becomes attached to the chain at exactly this moment [16], may then temporarily provide protection for the not yet folded, protruding polypeptide. γ-Crystallin, which has a slightly more resistant N-terminal sequence, would not require this extra protection.

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