NH$_2$-terminal Processing of Dictyostelium discoideum Actin in Vitro*

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When Dictyostelium discoideum actin is synthesized in a rabbit reticulocyte lysate system, it is made as a 43,000-dalton polypeptide with NH$_2$-terminal sequence Ac-Met-Asp-Gly ... even though in vivo the NH$_2$-terminal sequence is Ac-Asp-Gly ... (Rubenstein, P., Smith, P., Deuchler, J., and Redman, K. (1981) J. Biol. Chem. 256, 8149-8155). Here we describe the subsequent fate of the NH$_2$-terminal methionine residue of the actin synthesized in vitro. D. discoideum actin was synthesized in a rabbit reticulocyte lysate in the presence of L-[³⁵S]methionine. This fully translated and labeled actin was placed in a fresh aliquot of rabbit reticulocyte lysate under conditions where further protein acetylation was inhibited. This treatment resulted in the shift of the isoelectric point of actin to a more basic value characteristic of nonacetylated actin. Experiments using either [³⁵S]Met-tRNA$^\text{Met}$ as a sole source of label or tryptic and thermolytic digests of actin uniformly labeled with [³⁵S]methionine prove that this modification results from the removal of the NH$_2$-terminal acetyl and methionyl residues from the polypeptide chain to generate a polypeptide terminating in aspartic acid. Removal of the methionine apparently requires its prior acetylation. Once the NH$_2$-terminal acetic acid residue is exposed at the NH$_2$ terminus, the actin can again be acetylated in an acetyl-CoA-dependent reaction to yield a polypeptide probably identical with D. discoideum actin found in vivo. All of these reactions can occur post-translationally following release of the completed polypeptide chain from the ribosome. This series of experiments defines a pathway for actin NH$_2$-terminal processing that is different from the path used for other proteins studied so far.

Actin isolated from Dictyostelium discoideum, like all actins so far examined, has an acidic NH$_2$-terminal amino acid which is N-acetylated (2, 3). The NH$_2$-terminal amino acid sequence for this actin is Ac-Asp-Gly-Glu-Asp ... Nucleic acid sequencing of the D. discoideum actin gene (4) shows that the initiator methionine residue directly precedes the normal NH$_2$-terminal aspartic acid. Therefore, minimally, to produce the mature form of actin, the methionine must be removed from the new polypeptide allowing the aspartic acid to be acetylated.

For most other proteins studied, where methionine is remaining, it is required to be removed. A preliminary account of this work was presented at the 1981 American Society of Biological Chemists meeting in St. Louis (1). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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EXPERIMENTAL PROCEDURES

Materials—Materials for isoelectric focusing and gel electrophoresis were those described by O'Farrell (10). L-[³⁵S]Methionine (1000 Ci/mmol) was obtained from Amersham/Searle. Acetyl-CoA, pig heart citrate synthetase, cis-oxaloacetic acid, rabbit liver tRNA, creatine phosphokinase, hemin, and CoA were obtained from Sigma Chemical Co. Acetonyl-CoA was prepared according to the method of Rubenstein and Dryer (11). mRNA-dependent rabbit reticulocyte lysates were prepared according to Pelham and Jackson (12). D. discoideum whole cell RNA was obtained from cells of the Ax-3 strain grown in HL-5 medium by the procedure of Ulrich et al. (13). [³⁵S]Met-tRNA$^\text{Met}$ was prepared according to the procedures of Stanley (14) and Lenz and Baglioni (15). XAR-5 x-ray film was obtained from Eastman Kodak, and cellulose thin layer plates, 100 μm thick, were purchased from Eastman. L-1-Tosylamido-2-phenylethyl chloromethyl ketone trypsin and micrococcus nuclease were purchased from Boehringer. All other chemicals were reagent grade.

Cell-free Actin Synthesis—D. discoideum actin was synthesized in vitro using the mRNA-dependent rabbit reticulocyte lysate system of Pelham and Jackson (12) in the presence of [³⁵S]methionine or [³⁵S]Met-tRNA$^\text{Met}$ and D. discoideum whole cell RNA according to Rubenstein et al. (9). When the [³⁵S]Met-tRNA$^\text{Met}$ was used, 100 μCi unlabeled methionine was included to act as a trap for any [³⁵S]methionine released by hydrolysis of the labeled aminoacyl-tRNA. If we wished to inhibit endogenous protein acetylation in the lysate, it was first treated with citrate synthetase and oxaloacetate (16). Then, 75 μM S-acetyl-CoA was added with the mRNA (9). Reactions were analyzed by two-dimensional gel electrophoresis (10), and fluorograms of the gels were generated. The actin regions of the fluorograms were quantitated by scanning densitometry using a Transidyne RFT-II scanning densitometer.
NH₂-terminal Processing of Newly Translated Actin—At the end of a 45-min incubation a translation reaction described above was made 1 mM in unlabeled methionine and 100 μM in cycloheximide. This procedure terminated incorporation of labeled methionine into protein. The acetylation inhibition system described above was imposed, and a fresh aliquot of lysate also containing unlabeled methionine, cycloheximide, and the acetylation inhibition system was added as a source of enzymes. At desired times after incubation of the new reaction mixture at 25 °C, samples were withdrawn, and the actin was analyzed by two-dimensional gel fluorography as described above.

Peptide Mapping—Newly synthesized [³⁵S]methionine-labeled actin was isolated from translation reaction mixtures by DNase I-Sepharose chromatography as described by Zechel (17). The purified actin was then digested with performic acid and digested with tosylphenylalanyl chloromethyl ketone-treated trypsin using the method of Vandekerckhove and Weber (2). The digest was analyzed by electrophoresis on thin layer plates at pH 6.5, and the NH₂-terminal labeled tryptic peptide was identified with an authentic standard (9). The NH₂-terminal tryptic peptide was eluted and digested with thromlysin, and the digestion mixture was subjected to thin layer electrophoresis at pH 6.5. Autoradiograms were then made from the electrophoretograms.

RESULTS

Our recent work demonstrated that when D. discoideum actin was made in a rabbit reticulocyte lysate system in which endogenous protein acetylation was allowed to occur, the initial stable actin translation product had an acetylmethionine moiety instead of acetyl aspartic acid at its NH₂ terminus (9). To determine if we could produce a more mature form from this first stable intermediate, the acetylated actin uniformly labeled with [³⁵S]methionine ([U-³⁵S]Met-actin) was combined with fresh lysate under conditions where further translation and acetylation were blocked (see under “Experimental Procedures”). The actin was then analyzed by two-dimensional gel electrophoresis following various incubation times. The results are shown in Fig. 1. After 60 min a large percentage of the actin had shifted from its original position to a more basic one characteristic of nonacetylated actin (9, 18). Fully processed actin isolated from cells did not undergo this modification. One possible explanation was that the original acetyl group had been removed enzymatically during the second incubation; another was that both the acetyl and methionine residues had been removed. The removal of both would still result in an actin with one more positive charge than it originally had, and the loss of one amino acid would not be detected in the second dimension sodium dodecyl sulfate gel electrophoresis.

To distinguish between these possibilities, the experiment was repeated using [³⁵S]Met-tRNA⁰⁰ as a sole source of label in the initial translation system. Under these conditions only the NH₂-terminal methionine of actin would be labeled. If deacetylation alone had occurred, the labeled actin would merely move to a different position on the gel. If both the acetyl and methionine moieties were cleaved, the label would disappear from actin in the gel as processing was carried out. The results clearly showed that the label disappeared. The disappearance of the label as a function of incubation time is shown in Fig. 2 (black circles). Additional controls (data not shown) demonstrated that inhibition of acetylation during the reaction was not necessary for processing of the initiating methionine to occur.

The curves in Fig. 2 for removal of the initiator methionine (black circles) and for movement of uniformly methionine-labeled actin to the position of nonacetylated actin (triangles) were superimposable. This result suggested that acetylation might be required for removal of the initiator methionine. To test this possibility, actin labeled only at the initiator methionine was made in its nonacetylated form in vitro (see under “Experimental Procedures”). When this actin was combined with fresh lysate as described above, removal of the methionine occurred at about 16% the rate observed if the actin was acetylated. This processing is probably due largely to the actin that was acetylated initially even in the presence of the inhibition system (10–15%). For actin, as opposed to most other proteins, removal of the initiator methionine requires prior NH₂-terminal acetylation.

Peptide Map Studies—To confirm that both acetyl and methionine moieties were being removed from the initial fully translated actin polypeptide, we made use of the known trypsin and thermolysin cleavage sites in actin, as reported by Vandekerckhove and Weber (2). These protease cleavage sites are shown in Fig. 3. In the NH₂-terminal tryptic peptide, a single methionine is present in mature actin at position 16 and
an unique thermolysin cleavage site exists at Ala5. This thermolysin cleavage yields a highly acidic NH2-terminal (Nt) fragment and a much less acidic carboxylic (Ct) fragment of the original NH2-terminal tryptic peptide when electrophoresis is carried out at pH 6.5.

Fig. 4 demonstrates that when newly translated acetylated actin uniformly labeled with [35S]methionine is processed with fresh lysate, the NH2-terminal tryptic peptide moves to a position of lower mobility corresponding to one more positive charge than it originally had. This result shows that processing is occurring at the NH2 terminus and that at least the acetyl residue is being removed.

To gain information on the fate of the initiator methionine, the NH2-terminal tryptic peptides from processed and unprocessed actins labeled uniformly with [35S]methionine were cleaved with thermolysin, and the digestion products were subjected to electrophoresis at pH 6.5. The results are shown in Fig. 5. Curve A shows the labeled thermolytic cleavage products from the NH2-terminal tryptic peptide of unprocessed actin, and curve B shows the products from processed actin. It can be seen from Fig. 3 that if the initiator methionine is present, the Nt peptide will be labeled. If it is absent, the Nt peptide will be unlabeled. The result shown in Fig. 5 confirms that processing removes the initiator methionine from the NH2 terminus.

D. discoideum actin isolated in vivo contains acetyl-Asp at its NH2 terminus. Since the removal of acetyl-Met should leave an exposed aspartic acid amino group at the NH2 terminus we attempted to affect the acetylation of processed actin by incubating it with fresh lysate containing acetyl-CoA. The results, shown in Fig. 6, demonstrate that such an acetylation can occur. Controls show the reaction is absolutely dependent on acetyl-CoA. We have not rigorously proved that this second acetylation in the overall reaction sequence occurs at the NH2 terminus. However, this is the most probable site since, in the first acetylation, no internal lysine was acetylated.
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DISCUSSION

D. discoideum actin, when synthesized in a rabbit reticulocyte lysate, is made as a 43,000 molecular weight polypeptide which contains acetylmetionine at the NH$_2$ terminus (9). The results presented here show clearly that this initial stable translation product can be processed further, during which the acetyl and methionine moieties have been shown to be removed simultaneously. This hypothesis is reinforced by the increase in positive charge are identical during the course of the processing reaction. However, since the rate of removal of the initiator methionine and the rate at which actin increases in positive charge are identical during the course of the processing reaction, it is likely that these residues are removed simultaneously. This hypothesis is reinforced by the finding that acetylation is required before methionine cleavage occurs. If the acetylated methionine is deacetylated first, the original product would be regenerated. It is known to be a poor substrate for methionine removal. Direct chemical proof of this point must await the use of pure actin mRNA in the system, the methionine is removed after polymerization of the first 40 amino acids by a process which apparently does not require acetylation. If NH$_2$-terminal acetylation of the polypeptides subsequently occurs, it does so immediately following initiator methionine cleavage. Had actin been processed in this fashion it would have followed the path shown in Fig. 7, Scheme 2.

Most interesting is the fact that removal of the initiator methionine depends on its prior acetylation. For a number of proteins whose synthesis has been studied in the reticulocyte system, the methionine is removed after polymerization of the first 40 amino acids by a process which apparently does not require acetylation. If NH$_2$-terminal acetylation of the polypeptides occurs, it does so immediately following initiator methionine cleavage. The results presented here show clearly that this initial stable translation product can be processed further, during which the acetyl and methionine moieties have been removed simultaneously. This hypothesis is reinforced by the increase in positive charge are identical during the course of the processing reaction. However, since the rate of removal of the initiator methionine and the rate at which actin increases in positive charge are identical during the course of the processing reaction, it is likely that these residues are removed simultaneously. This hypothesis is reinforced by the finding that acetylation is required before methionine cleavage occurs. If the acetylated methionine is deacetylated first, the original product would be regenerated. It is known to be a poor substrate for methionine removal. Direct chemical proof of this point must await the use of pure actin mRNA in the system, the methionine is removed after polymerization of the first 40 amino acids by a process which apparently does not require acetylation. If NH$_2$-terminal acetylation of the polypeptides occurs, it does so immediately following initiator methionine cleavage. Had actin been processed in this fashion it would have followed the path shown in Fig. 7, Scheme 2.

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Actin may be one of a small class of proteins processed in this fashion. Another possible example is rabbit fast skeletal muscle troponin C which has Ac-Asp-Thr-Glu- at its NH$_2$ terminus (20). Whether this protein is processed like actin depends on whether or not the initiator methionine directly precedes the NH$_2$-terminal aspartate. This information requires sequencing the fast muscle troponin C gene which, to our knowledge, has not been done. Interestingly, troponin C from slow skeletal muscle or cardiac muscle has the NH$_2$-terminal sequence Ac-Met-Asp-Ile (21). The methionine here has been retained, as would be expected on the basis of studies of other proteins.

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REFERENCES