THE EFFECT OF PROTEOLYSIS ON THE STABILITY OF THE PROFILACTIN COMPLEX

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1. Introduction

There is evidence for conversion of unpolymerized to filamentous actin occurring in parallel with changes in morphology and motile behaviour of cells stimulated from without [1,11]. Analysis of platelet extracts reveals that stimulation of the platelets with thrombin leads to a decrease in the amount of profilactin and a simultaneous increase in the amount of free profilin (F. Markey and T. Persson, unpublished). This implies that profilactin is a precursor to filamentous actin. The factors involved in and controlling this process are unknown.

Binding of profilin to actin prevents the actin from forming filaments under conditions where G-actin alone will polymerize rapidly. We report here that tampering with the C-termini of profilactin results in dissociation of the complex allowing the actin to polymerize.

2. Materials and methods

Profilactin from calf spleen was prepared as described in [2]. Carboxypeptidase A (DFP-treated and twice crystallized) was purchased from Sigma Chemical Company. The carboxypeptidase A digestion was carried out essentially as described in [3] but using 5 mM potassium phosphate buffer pH 7.6, 0.1 mM $CaCl_2$, 0.1 mM ATP, 0.1 mM DTE. Digestions were also performed under actin polymerizing conditions (15 mM NaCl and 3.5 mM $MgCl_2$) in the buffer as above. For the identification of amino acids released from profilactin by carboxypeptidase aliquots were withdrawn from the reaction mixture at intervals during incubation. The samples were immediately frozen at −80°C, freeze-dried and dissolved again later and analyzed from free amino acids using a Beckman 121-M amino acid analyzer. Nor-leucine was added to the reaction mixture as internal standard. Viscometry was performed at 37°C using a Canon-Manning semi-micro capillary viscometer requiring a volume of about 0.7 ml, and having a flow time for buffer of 60 s. Sedimentation of filamentous actin was carried out in a Sorvall OTD-2 ultracentrifuge using the AH-650 swing-out rotor at 100 000 × *g* for 3 h. Electrophoresis in SDS-polyacrylamide gels was performed as in [4]. Actin filaments were visualized by electron microscopy after fixation with 2% glutaraldehyde and negative staining using 2% sodium silicotungstate. The samples were examined in a Jeol 100C electron microscope at 80 kV. The DNase I inhibition assay was performed as described in [5]. Protein concentrations were determined spectrophotometrically using the following values for $E^{1\%}_{280\ nm}{}^{1\ cm}$ actin 11.0, profilin 13.0, and profilactin 11.0.

3. Results and discussion

Our experience with profilactin from calf spleen has been that it varies in stability from preparation to preparation. We find that some preparations are relatively stable whereas others give rise to actin filaments after short times of incubation in the viscometer. The reason for this variability is unclear. There could be variable amounts of (a) contaminating factor(s) in the profilactin preparations which somehow modifies the complex so that it dissociates with polymerization of the actin as a result. We have considered the possibility that limited proteolysis could contribute to destabilization of the complex. Native actin is notoriously resistant towards digestion with trypsin and chymotrypsin and Tilney has shown that trypsin digestion leads to the appearance of actin filaments in preparations of red cell membranes and release of unpolym-

erized actin from its storage form in the subacrosomal cup of Thyone sperm [6,7].

Exposure of profilactin to trypsin (substrate to enzyme radio 200:1 w/w) in 5 mM potassium phosphate buffer pH 7.6, 15 mM NaCl, 3.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM ATP, 0.1 mM DTE results in polymerization of the actin. Analysis by SDS-polyacrylamide gel electrophoresis demonstrated that there were no changes in the mobility of the two polypeptides (profilin and actin), but analysis of the digests by gel chromatography revealed the presence of low MW material. This material was shown by 2D peptide mapping and amino acid analysis of isolated peptides to consist of a number of fragments emanating from the C-terminal end of profilin and from both N- and C-terminal ends of actin (data not shown).

To characterize more closely which parts of profilin and/or actin are essential to the stability of the complex the effect of carboxypeptidase A on profilactin was investigated. In the experiment described in fig.1 profilactin at a concentration of 0.9 mg/ml was incubated with carboxypeptidase A at a substrate to enzyme ratio of 100:1. Panel A shows the viscosity of the incubation mixture when carboxypeptidase A was added in the presence of salt to allow actin polymerization from the beginning of the experiment. After a lag period of 8–10 min the viscosity of the sample began to increase indicating the formation of actin filaments. In this experiment 65% of the actin formed filaments which could be sedimented by high speed centrifugation. This is in good agreement with the extent of polymerization estimated from the final level of viscosity and with the value of 63% F-actin obtained with the DNase inhibition assay for unpolymerized and polymerized actin (data not shown). If the profilactin was preincubated with the carboxypeptidase A under non polymerizing conditions there was no lag phase in the polymerization seen after salt addition (Panel B) suggesting that free polymerizable actin had accumulated during the preincubation with the enzyme (see below).

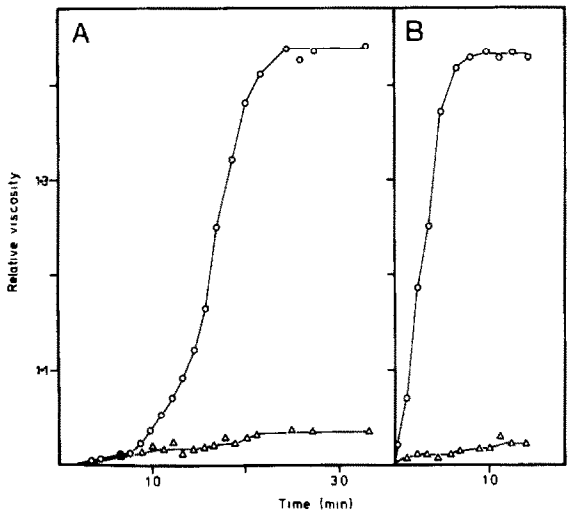

Fig.1. The polymerization of actin from profilactin induced by carboxypeptidase A. Polymerization of the actin was followed by viscometry in 5 mM potassium phosphate buffer pH 7.6, 0.1 mM $CaCl_2$, 0.1 mM ATP, 0.1 mM DTE as described in Section 2. The concentration of profilactin 0.91 mg/ml that is 0.68 mg of actin per ml. (A) Viscosity increase following the addition of carboxypeptidase A and salt at the same time (open circles); the triangles show the viscosity of a polymerization mixture minus carboxypeptidase A; (B) viscosity of a sample incubated with carboxypeptidase A for 30 min at 37°C before the addition of salt (15 mM NaCl, 3.5 mM $MgCl_2$).

Fig.3A illustrates the analysis of the products of carboxypeptidase A digestion by SDS-polyacrylamide gel electrophoresis. The results show (i) that there is no change in the mobility of the two polypeptides following carboxypeptidase A-digestion, and (ii) that

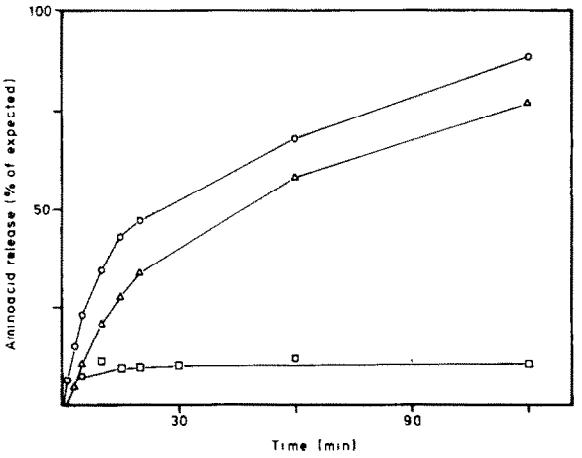

Fig.2. The time course of amino acid release from profilactin caused by carboxypeptidase A. Profilactin was incubated at 37°C in 5 mM potassium phosphate buffer pH 7.6, 0.1 mM $CaCl_2$, 0.1 mM ATP, 0.1 mM DTE. Samples were removed from the incubation mixture and analyzed for free amino acids as described in Section 2. Release of: ○, phenylalanine; △, tyrosine; □, serine/glutamine. Serine and glutamine were not separated on the amino acid analyser.

the material which sediments at high speed centrifugation is actin alone and that all the profilin is left in the supernatant together with non sedimentable actin. Fig.3B is an electron micrograph of pelleted material showing the actin filaments formed after the carboxypeptidase digestion.

Chromatography of the products from digestion of profilactin under non-polymerizing (low salt) conditions on Sephadex G-100 resulted in a quantitative separation of profilin and actin demonstrating that polymerization of the actin is not a prerequisite for the separation of the two proteins (data not shown). We do not know why the actin is not quantitatively converted to filaments under polymerizing (high salt) conditions. It is possible that the critical concentration for polymerization of the actin under the conditions of the experiment is higher than expected, or that for some reason part of the actin is modified in such a way that it has lost the ability to form filaments.

To investigate which amino acids were released, aliquots were removed from the incubation mixture during the carboxypeptidase A-induced actin polymerization. Knowing the specificity of carboxypeptidase A and the amino acid sequence of the two proteins one would expect phenylalanine to be the only amino acid released from the C-terminus of actin [8,9] and tyrosine, glutamine and lower amounts of serine from profilin [10]. As shown in fig.2 phenylalanine and tyrosine were the two residues released in largest amounts, with recoveries approaching 100%. Both glutamine and serine were released in unexpectedly low amounts considering the known specificity of the enzyme.

There was also a release of smaller amounts of other amino acids, but when the digestion of the complex was performed in 6 M urea (carboxypeptidase A is till active in this milieu) the release of the additional amino acids was abolished (data not shown). This seems to suggest that other proteases (inactive in 6 M urea) contaminate the carboxypeptidase preparation and introduce nicks into the polypeptide chains in the absence of urea, thereby providing additional C-terminals where the carboxypeptidase A can act.

It is clear from the experiments presented here that only a small change in profilactin is enough to cause dissociation of the complex. Whether it is removal of one or the other or both of the C-terminal amino acids which causes the dissociation has not yet been determined, but should be possible to investigate by recombination experiments using the isolated native proteins together with the modified variants.

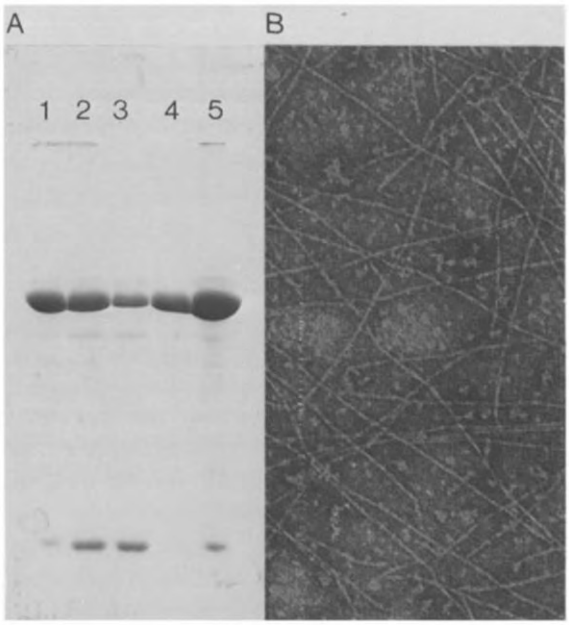

Fig.3. The analysis of the products from the profilactin carboxypeptidase A interaction by gel electrophoresis and electron microscopy. (A) SDS-polyacrylamide gel electrophoresis of slot (1) profilactin before digestion; (2) profilactin after 40 min of incubation with carboxypeptidase A; (3) supernatant after digestion, polymerization and ultracentrifugation to sediment actin filaments; (4) pelleted material from the experiment in (3); and (5) profilactin before digestion with the enzyme. (B) Electron micrograph of pelleted material from a sample of profilactin treated with carboxypeptidase A under polymerizing conditions. Magnification 73 000 ×.

The experiments presented here were initiated because we wanted to find out if proteolysis could be involved in causing the variations seen in the stability of profilactin. They have demonstrated clearly that it is of utmost importance to avoid proteolysis during the purification of profilactin and provided a method by which the quality of the product can be assessed. Intact profilactin should yield a stoichiometric amount of phenylalanine and tyrosine upon digestion with carboxypeptidase A in 6 M urea, and no other amino acids released. We have analyzed unstable preparations of profilactin before and after spontaneous polymerization using this methodology and obtained a full recovery of the two C-terminal amino acids.

Small amounts of additional amino acids were seen, but in amounts suggesting that only about 5% of the material contained internal nicks. Thus limited proteolysis does *not* seem to be the prime reason for instability of the profilactin complex in these experiments, but rather some other still unidentified factor. The carboxypeptidase results are important however, in that they seem to suggest that the C-terminal regions of profilin and/or actin contains sensitive structures governing the stability of the profilactin complex.

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