Chemical modification of Cys-374 of actin interferes with the formation of the profilactin complex

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Chemical modification of the cysteine residue 374 of actin, both with *N*-ethylmaleimide and with the fluorescent probe *N*-(1-pyrenyl)iodoacetamide, is shown to counteract the inhibiting effect of profilin on actin polymerization.

Actin *Profilin* *Profilactin* *Pyrenyl actin* *Actin polymerization* *Sulfhydryl group*

1. INTRODUCTION

The C-terminal phenylalanine of actin is of the utmost importance for the interaction between profilin and actin, in that removal of the phenylalanine appears to abolish the interaction between the two proteins [1]. In juxtaposition to the phenylalanine is a cysteine residue which is known to react with different organic and inorganic compounds, some of which are fluorescent and used as probes to monitor the polymerization of actin. Since these probes are sensitive and useful tools in the study of actin polymerization, it was of interest to see if native actin and actin labelled at the C-terminal cysteine differed in their ability to recombine with profilin. Recombination experiments with a chemically modified actin could also provide further evidence of the importance of the C-terminal of actin in its interaction with profilin.

This paper presents evidence that chemical modification of Cys-374 of actin eliminates the inhibitory effect of profilin on the polymerization of actin. One of the reagents used to modify the C-terminal of actin was the fluorescent probe *N*-(1-pyrenyl)iodoacetamide. It is indicated here that actin modified in this way does not recombine with spleen profilin.

2. MATERIALS AND METHODS

Profilactin from calf spleen was prepared essentially as in [2]. For the preparation of *N*-ethylmaleimide (NEM)-labelled actin, profilactin (10–15 mg/ml) was mixed with an equal volume of 2 M potassium phosphate (pH 7.6), 6 mM $MgCl_2$, 4 mM ATP, 2 mM DTT and the mixture was incubated for 1–2 h at 25°C. The actin paracrystals which then formed were collected by centrifugation for 30 min at 20000 × *g* and 15°C. To remove contaminating proteins, the paracrystals were suspended in 5 mM potassium phosphate (pH 7.6), 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.5 mM ATP, 2 mM DTT and the dispersed filaments pelleted by centrifugation for 4 h at 100000 × *g* and 15°C. One-half of the pelleted material was homogenized in 5 mM potassium phosphate (pH 7.6), 0.2 mM $CaCl_2$, 0.5 mM ATP, 2 mM DTT and dialyzed against the same buffer for 48–72 h. The dialyzed sample was then centrifuged for 3 h at 100000 × *g* and 4°C to obtain monomeric actin. The other half of the sedimented F-actin was suspended in 5 mM potassium phosphate (pH 7.6), 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.5 mM ATP. NEM was added to a molar ratio of 20:1 (NEM:actin) and the sample incubated at 4°C for 20 h. To stop the reaction 5-fold molar excess of DTT over NEM was added.

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The labelled actin was pelleted by centrifugation for 4 h at 100000 × *g*, 15°C and depolymerized as described above.

To prepare the pyrene-labelled and control actin used in the experiment described in fig.2, actin was polymerized from profilactin as described above. The pellet of washed F-actin was suspended in 10 mM potassium phosphate (pH 7.6), 50 mM KCl, 1 mM $MgCl_2$ and 1 mM ATP. To one-half of the suspension a 7-fold molar excess of *N*-(1-pyrenyl)iodoacetamide (Molecular Probes, OR) (dissolved in dimethylformamide) was added [3]. Both halves of the suspension were incubated at 4°C for 16 h with continuous stirring, after which the actin was collected by centrifugation for 3 h at 100000 × *g* at 4°C and depolymerized as described above. Both the pyrene-labelled and control actin were chromatographed on a Sephadex G-100 superfine column to obtain monomeric actin.

The profilin used in these experiments contained C-terminal tyrosine and was prepared as in [1]. The profilactin 'stabilizing factor' and the 'factor-free' actin used in the control experiment described in fig.1B were prepared as in [1]. Viscometry was performed at 25°C using a Cannon-Manning semimicro capillary viscometer requiring a sample volume of 0.7 ml and having a flow time for buffer of about 60 s. Fluorescence measurements were carried out on an Aminco-Baumann SPF 500 spectrophotofluorometer.

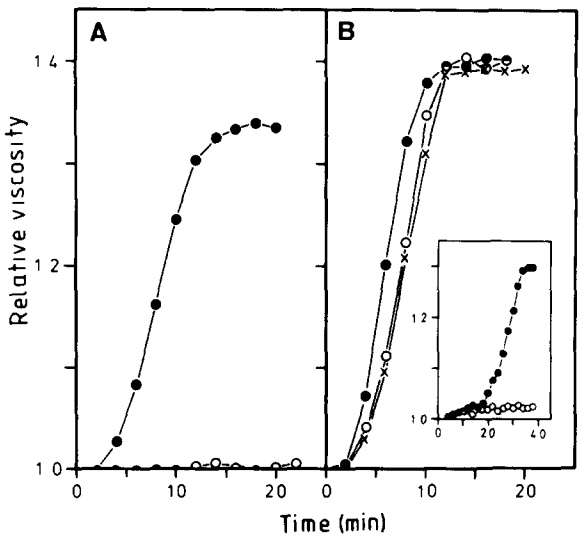

Fig.1. Recombination of profilin with NEM-labelled actin. Actin (0.35 mg) was incubated alone or with profilin (0.14 mg, giving a molar ratio of actin to profilin of 1:1.1) in a total volume of 0.7 ml for 10 min. At zero time, 7 μl of 200 mM $MgCl_2$ was added to give a final concentration of 2 mM and the polymerization of the actin was followed by viscometry. (A) Unlabelled actin (●), unlabelled actin + profilin (○). (B) NEM-labelled actin (●), NEM-labelled actin + profilin (○), NEM-labelled actin + profilin + 0.1 ml of a fraction containing the profilactin stabilizing factor (×). The inset demonstrates the effect of the same amount of the factor on the polymerization of a factor-free actin recombined with profilin under the same conditions as described above. (●) Actin + profilin, (○) actin + profilin + factor.

3. RESULTS AND DISCUSSION

In the experiment described in fig.1 unlabelled and NEM-labelled actin, prepared from the same batch of profilactin, were recombined with profilin and checked for polymerization as described in the figure legend. Panel A shows the polymerization of unlabelled actin in the absence and presence of profilin. The addition of profilin under these conditions totally counteracted the polymerization of the actin during the subsequent time period. Panel B shows the same type of experiment but with the chemically modified actin. In this experiment the profilin had only a small effect on the polymerization of the actin.

As described in [1], actin prepared from spleen profilactin is contaminated with variable amounts of a factor causing an apparent stabilization of the complex. In the method used for preparing actin from profilactin small amounts of the factor copurify with the actin, which is illustrated by the behaviour of the recombined complex in fig.1A. A more extensively purified actin recombined with profilin polymerizes after a lag period of about 15 min (see fig.2A). To determine whether the effect of the NEM-labelling was caused by inactivation of the factor, an aliquot from a fraction containing the stabilizing factor was added to NEM-labelled actin recombined with profilin (fig.1B). The addition of the factor had almost no effect on the polymerization of the labelled actin, whereas this amount of factor added to a mixture of profilin and unlabelled factor-free actin at the same protein concentrations and conditions as in the rest of the experiment caused a complete stabilization

of the complex as illustrated in the inset of fig.1B. Thus labelling of Cys-374 of actin with NEM eliminates the effect of profilin on the polymerization of actin.

In recent years a new method for studying actin polymerization has been developed. The method is based on coupling a fluorescent label to the C-terminal cysteine residue of actin. The increase in fluorescence, obtained when the actin is polymerized, is directly proportional to the amount of labelled actin incorporated into filaments. The labelled actin has similar characteristics of polymerization and critical concentration to unlabelled actin and in mixtures copolymerizes with native actin [3,4]. If the effect of profilin on the polymerization of actin were unaffected by the labelling it would also be useful in the study of profilin-actin recombination.

Fig.2 shows a recombination experiment using actin labelled to 30% with *N*-(1-pyrenyl)iodoacetamide. Panel A shows the behaviour of actin recombined with profilin in the presence of 2 mM $MgCl_2$. Again there was a difference between

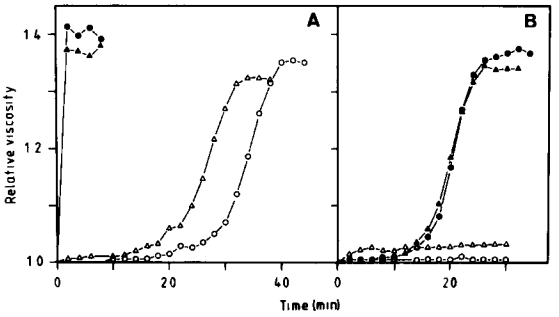

Fig.2. Recombination between profilin and pyrene-labelled actin monitored by viscometry. (A,B) Actin (unlabelled or pyrene-labelled to 30%) (0.24 mg) was incubated alone or with 0.1 mg profilin (1:1.1 molar ratio of actin to profilin) in a total volume of 0.7 ml. (A) Samples were incubated for 10 min, after which $MgCl_2$ (2 mM final concentration) was added to initiate polymerisation. (B) Samples containing actin alone were incubated as described above but KCl (100 mM final concentration) was added at zero time. Samples containing actin and profilin were incubated for 10 min in the presence of 100 mM KCl after which sonicated F-actin (10 μl sonicated unlabelled actin polymerised in 100 mM KCl) was added at zero time. The polymerisation of actin was followed by viscometry. Unlabelled actin (●), unlabelled actin + profilin (○), labelled actin (▲), labelled actin + profilin (△).

labelled and unlabelled actin although less pronounced than in fig.1. The experiment was repeated but with 100 mM KCl as polymerizing agent (panel B). Under these conditions the dissociation constant for spleen profilactin (determined with the viscosimetric assay of [5]) is $\leq 4 \times 10^{-8}$ M and the critical concentration for the polymerization of actin is 1.4×10^{-6} M (H. Larsson, personal communication). Thus unlabelled actin gives a stable complex with profilin in this milieu, and no polymerization occurs even when small amounts of sonicated F-actin are added to overcome the lag phase due to the relatively slow nucleation process in the polymerization. This is illustrated in fig.2B, where no increase in viscosity is seen in the case of unlabelled actin mixed with profilin. However, with the pyrene-labelled actin preparation mixed with profilin, a rapid but small increase in viscosity was seen when nuclei were added. Thus pyrene labelling of actin weakens the inhibitory effect of profilin on the viscosity increase. The final level of viscosity in this case was 1.04, which corresponds to the level of polymerization obtained with pure spleen actin at a concentration of 0.09 mg/ml. This concentration is close to that of the pyrene-labelled actin present in the experiment (0.10 mg/ml).

Fig.3 illustrates the increase in fluorescence intensity when the pyrene-labelled actin (same preparation as in fig.2) with and without profilin was polymerized by the addition of 100 mM KCl and nucleating amounts of sonicated F-actin. The plateau value for fluorescence in the recombination mixture indicates that 90% of the labelled actin has polymerized, although viscosimetry suggested that only 30% of the total actin had formed filaments. Since the degree of modification in this actin preparation was 30%, the simplest interpretation of these results is that the increase in viscosity in fig.2B was due to an almost complete polymerization of only the pyrene-labelled part of the actin preparation, suggesting that profilin does not recombine with the modified actin.

In [6] data are presented showing that profilin from *Acanthamoeba castellanii* recombines with pyrene-labelled *Acanthamoeba* actin. Actin from *Acanthamoeba* and spleen are closely similar [7,8] but the *Acanthamoeba* and spleen profilins differ considerably in molecular mass, amino acid composition and circular dichroism spectra [1,9–12].

The present experiments suggest that the profilins from the two sources also differ in their interaction with actin.

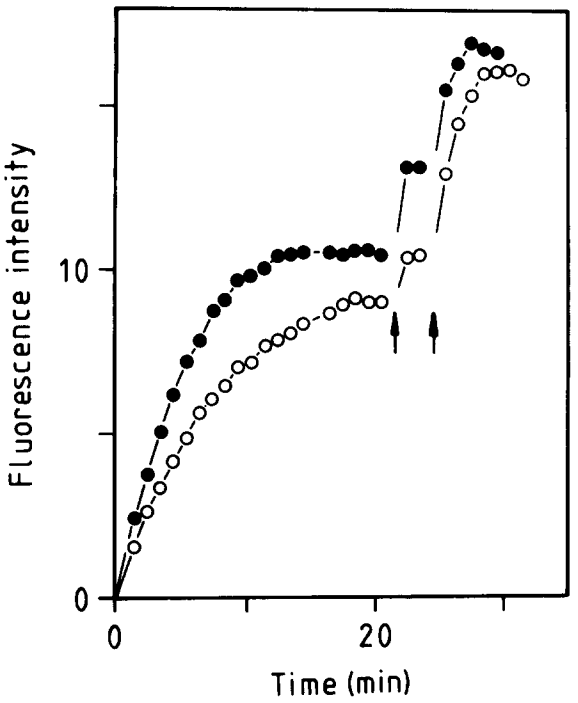

Fig.3. Recombination between profilin and pyrene-labelled actin monitored by fluorometry. Actin, pyrene-labelled to 30% (0.35 mg), was incubated alone or with 0.14 mg profilin (giving an actin:profilin molar ratio of 1:1.1) in a total volume of 1 ml containing 100 mM KCl for 6.5 min. Sonicated F-actin (15 μl of a sample prepared as in fig.2B) was added at zero time and the polymerisation of actin was monitored as the increase in fluorescence (excitation, 365 nm; emission, 407 nm). At 21 min (arrow) the cuvettes were taken out from the fluorometer and gently shaken by hand. At 24 min (arrow) $MgCl_2$ was added (2 mM final concentration). Pyrene-labelled actin alone (●), pyrene-labelled actin + profilin (○).

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