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Probing the phalloidin binding site of actin

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Phallotoxins form tight complexes with filamentous actin and stabilize the polymer against shearing stress. In the present study a phalloidin derivative containing a thiol-capturing moiety was prepared and reacted with single thiol groups of monomeric muscle actin. Sites of attachment in the protein were Cys-374 next to the C-terminus and Cys-10, close to the N-terminus; the latter was recently shown to be uncovered during a slow but reversible conformational transition occurring in ADP–G-actin. Phalloidin bound to Cys-374 stabilizes filaments against shearing stress almost as effectively as free phalloidin, indicating that the phalloidin binding site cannot be far from the C-terminus of actin. Stabilization was also achieved when the phalloidin reagent was added to F-actin, however, the subsequent formation of a covalent linkage with Cys-374 was not observed, most likely due to a restricted mobility of the reactants. In contrast to the efficient stabilization of filaments by phalloidin linked to Cys-374 a destabilizing effect was observed when phalloidin was attached to Cys-10. It appears that phalloidin located close to the N-terminus is unable to bind to the normal binding site in its own filament. Pronounced gelification of this actin derivative suggests that the toxin is able to mediate crosslinking with neighbouring filaments. From these results we conclude that the phalloidin binding site of actin is distant from the N-terminus, but close to the C-terminus. Furthermore, the data provide evidence that binding of phalloidin reduces the mobility of the C-terminus.

Muscle actin; Phalloidin binding site; Actin thiol; Covalently linked phalloidin; Steady-state ATPase; Filament stabilization

1. INTRODUCTION

Phalloidin, a cyclic peptide isolated from *Amanita phalloides* mushrooms, binds to filamentous actin with high affinity. The binding of phalloidin lowers the critical concentration of actin by a factor of up to 30 [1], due to a decrease in the rate constants for the dissociation of actin subunits from the filament ends [2]. The decreased critical concentration is reflected in a higher stability of the filaments, for example against shearing stress. The stoichiometry of binding is probably one phalloidin for one actin protomer [3]. Nevertheless, full stabilization may be achieved even with substoichiometric amounts of the toxin (as shown, for example, in a pelleting assay [4]). This is in line with our recent finding that the activity of phalloidin is characterized by a high degree of cooperativity [5]. We were able to show that full decrease of steady-state ATPase activity in actin filaments was reached with only one out of 3 subunits complexed by the toxin.

Using photoactivatable phalloidin derivatives it was shown that the toxin is bound close in the vicinity to Glu- 117, Met-119 and Met-355, leaving open the question of whether phalloidin is in contact with one, or more than one, subunit in the filament [6]. With elucidation of the atomic models of G-actin [7] and F-actin [8] it became known that all amino acid residues that were affinity-labeled with the phalloidin derivative are located in subdomain 1. Likewise located in subdomain 1 are the two cysteine residues of muscle actin, which are accessible to specific modification under defined conditions, namely Cys-374 in the ATP complex, and Cys-10 in the ADP complex [9]. Since these two cysteine residues are located on opposite sides of the molecule [7], they can be used in determining on which side phalloidin is bound. For this purpose we covalently attached a phallotoxin to these positions using an activated asymmetric disulfide of the type shown in Fig. 1. Reagents of this type can be prepared very easily, and will allow thiol compounds to be introduced into thiol-exposing proteins, with the advantage of monitoring the reaction by spectrophotometry [10–16].

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2. MATERIALS AND METHODS

2.1. *Reagents*

All reagents were of analytical grade and all solutions were prepared with double-distilled water. ATP and ADP were from Pharma-Waldhof (Düsseldorf, Germany); [ethylene-bis(oxyethylenenitrilo)]-tetraacetic acid (EGTA), *N*-ethylmaleimide (NEM), *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), hexokinase (C-302) and DNase I were from Sigma, and Sephadex G-25 from Pharmacia LKB Biotechnology Inc. Aminomethyldithiolano-phalloidin and *n*-octyl-5-dithio-2-nitrobenzoic acid (ODNB) were preparations of our own laboratory.

ADP was purified from traces of contaminating ATP by ion-exchange chromatography on QAE-Sephadex C-25 as described [11].

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2.2. *Preparation of the phalloidin reagent*

Aminomethyl-dithiolano-phalloidin (Fig. 1) was prepared as described [22,23] and used as a mixture of the 4 isomers. 20 mg of this phalloidin derivative was dissolved in 3 ml H_2O and adjusted to pH 8 by the addition of 0.1 N NaOH (ca. 0.04 ml). To this solution, 50 mg of SPDP, dissolved in 0.6 ml were added and reacted for 1 h under magnetic stirring. During this time, pH was kept at pH 8.0 by the addition of small portions of 1 N NaOH (in total 0.07 ml). The reaction mixture was then applied to three analytical grade silica thin layer plates (Merck HF_{254}, 20 × 20 cm) and developed with chloroform–methanol–water (65:25:4). The reaction products (2 bands at R_f = 0.64 and 0.70, visible in UV light and producing a blue color when reacted with cinnamic aldehyde-HCl), were scraped off, eluted with methanol and evaporated in vacuo. For complete removal of SiO_2 the substance was filtered through a small column of Sephadex LH 20 (1.8 × 30 cm) developed with methanol. Yield, 9 mg.

The phalloidin derivative was characterized by its UV spectrum as well as by the release of a stoichiometric amount of thiopyridine upon the addition of 10 μl β-mercaptoethanol (Fig. 2).

2.3. *Preparation of actin*

Actin from rabbit skeletal muscle was prepared from acetone powder essentially as described [17] with an additional Sephadex G-150 gel filtration step, and was pure as checked by SDS-PAGE. G-Actin was used immediately after gel filtration. Blocking of Cys-374 with NEM was achieved by incubation of G-actin with a 50-fold excess of NEM in buffer 1 (2 mM Tris-HCl, pH 7.8, containing 0.2 mM ATP and 0.1 mM $CaCl_2$) at room temperature. The reaction was quenched after 30 min by adding excess dithiothreitol and separation of the protein on a small Sephadex G-25 column eluated with buffer 1. F-Actin was prepared by the addition of EGTA/$MgCl_2$ to final concentrations of 0.2 and 1 mM, respectively. Removal of the ATP was achieved by incubation with hexokinase (to a final concentration of 5 U/ml actin) and glucose (to 0.4 mM) for 1–2 h [18]. Actin was sedimented by ultracentrifugation and the pellets allowed to soften on ice for a few hours in buffer 2 (2 mM Tris-HCl, pH 7.8, containing 1 mM ADP and 1 mM NaN_3) and then homogenized gently in a Teflon/glass potter. The resulting solution of monomeric ADP–actin was clarified by ultracentrifugation at 300,000 × *g* for 1 h at 4°C before use. Protein concentrations of G-actin solutions were determined spectrophotometrically using ε_{290} = 26,460 $M^{-1} \cdot cm^{-1}$ and a molecular mass of 42,300 Da.

2.4. *Determination of the relative affinity constant for the phallotoxin reagent*

Relative affinity of the phalloidin reagent to actin was determined by the release of a tritiated phallotoxin from the F-actin complex by incubation with increasing amounts of the reagent. The procedure is described in detail in [19].

2.5. *Reaction of exposed thiols with the phallotoxin reagent*

The reaction was started adding a 10-fold excess of reagent (as a 20 mM solution in ethanol) to sample and reference cuvettes, and followed spectrophotometrically at 343 nm using ε_{343} = 8,080 $M^{-1} \cdot cm^{-1}$ [20]. The spectrometer Aminco DW-2 was equipped with a thermostatic cell compartment cooled to 4°C.

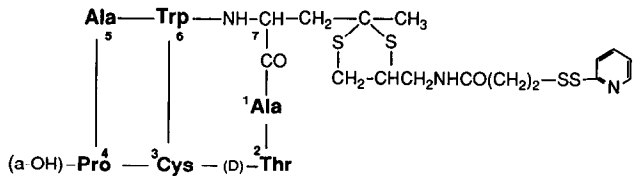

Fig. 1. Structure of the thiol-capturing phalloidin derivative used in this study.

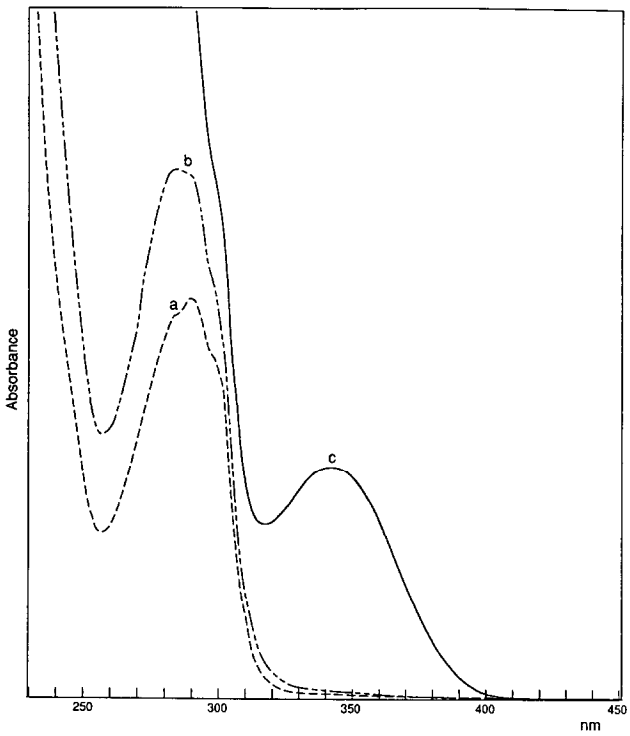

Fig. 2. UV-spectra of aminomethyldithiolano-phalloidin (a) and 2-pyridyldithiopropionyl-aminomethyl-dithiolano-phalloidin without (b) and with (c) added dithiothreitol.

2.6. *Polymerization measurements*

Polymerization was followed at 25°C by the enhancement of light scattering at 400 nm in an SLM 4000 spectrofluorimeter equipped with a thermostated cell compartment. Alternatively, we monitored polymerization by determining relative viscosity values in a Cannon capillary viscosimeter as described previously by [12]. In both cases polymerization was induced by addition of $MgCl_2$ up to a final concentration of 2 mM.

2.7. *Steady-state ATPase*

Steady-state ATPase activity of filamentous actin was measured as reported in [14].

3. RESULTS

The phallotoxin derivative containing the thiol sequestering moiety (Fig. 1) was characterized via its ultraviolet absorption spectrum (Fig. 2), using the extinction coefficients of the pure toxin (ε_{300} = 10,100 $M^{-1} \cdot cm^{-1}$) and of the toxin derivative (ε_{300} = 11,500 $M^{-1} \cdot cm^{-1}$); the amount of pyridyl-2-thiolate released on the addition of mercaptoethanol was also determined (ε_{343} = 8,080 $M^{-1} \cdot cm^{-1}$). This analysis indicated that the toxin derivative contained the phallotoxin and the 2-pyridyldisulfide moiety in a ratio of 1:0.9. Due to the linker moiety introduced, the toxin derivative showed a somewhat reduced affinity for actin, as monitored by its capacity to displace [^{3}H]demethylphalloin from its binding site in filamentous actin. The relative

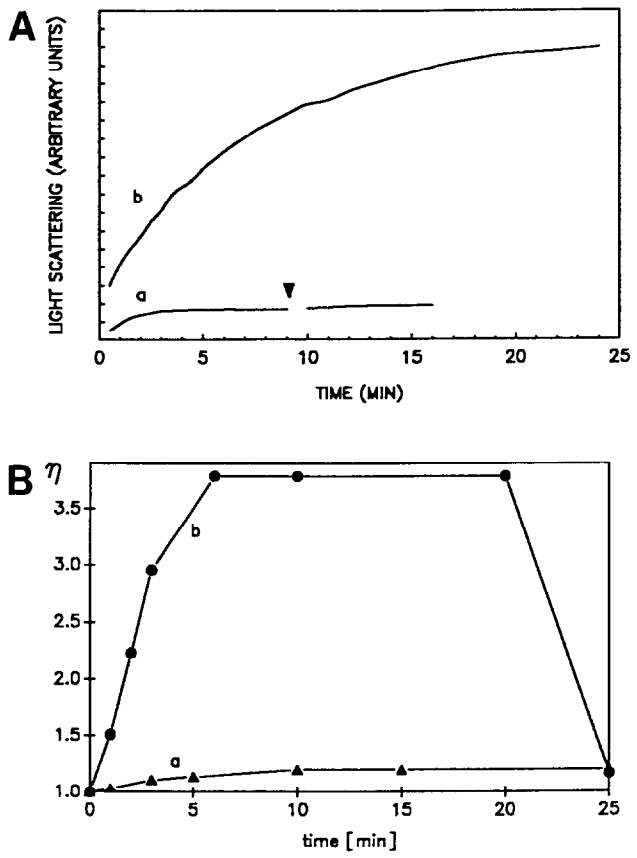

Fig. 3. Polymerization kinetics of the 2-pyridyldithiopropionyl-aminomethyldithiolano-phalloidin derivatives of G-actin modified at position 374 (a) and 10 (b), monitored by light scattering (A) and viscosimetry (B). The values of curve b in B were obtained by using separate actin samples for each point. Only the penultimate sample was measured twice, showing the complete and irreversible liquification of the gel by one passage through the capillary. In A one equivalent of phalloidin was added at the arrowhead.

affinity (phalloidin = 100%) was 87%. The affinity was measured in the presence of excess β-mercaptoethanol in order to remove the thiopyridyl residue and prevent the formation of a covalent link between actin and the phallotoxins.

Spectrophotometric control at 343 nm showed that complete derivatization of Cys-374 of actin required ca. 3 h at 4°C, when a 5-fold molar excess of the reagent over the protein was used. Derivatization of Cys-10 in ADP-actin, in which Cys-374 had been previously blocked with NEM, was faster, with a duration of ca. 30 min under the same conditions. After separation from excess reagent by gel filtration the covalent attachment of the toxin was assessed in the following way: a sample was precipitated with 10% trichloroacetic acid, and it was shown by spectrophotometry that no phallotoxin was present in the supernatant. A second sample was incubated with dithiothreitol (DTT) before precipitation; from the toxin concentration in the supernatant we determined that the extent of binding was about 90% for Cys-374, and 70–80% for Cys-10.

The Cys-374-, as well as the Cys-10 derivative of actin was polymerizable in the presence of 1 mM $MgCl_2$. Polymerization was monitored by viscosimetry and light scattering. In both systems the Cys-10 derivative showed irregular behavior because of rapid gelification. Formation of a network is suggested by values of both light scattering and viscosity, which were, respectively, 8-fold (Fig. 3A), and 2-fold (Fig. 3B); higher than those of normal actin in the presence of phalloidin. (Viscosity measurements were possible only by using a very low concentration of actin, 5.7 μM, and separate samples for each point on the time axis.) The gel formed from the Cys-10 derivative was extremely susceptible to shearing stress: mechanical stirring or a single run through the capillary (Fig. 3B) liquidified the gel irreversibly and reduced the η-values to those of normal F-actin. This may explain why, under the electron microscope, the majority of the gel consisted of filaments that were similar to those from normal actin (data not shown).

The filament-stabilizing capacity of an actin-binding agent can be measured by its inhibitory effect on the steady-state ATPase activity of a polymeric actin solution [13]. This method was applied in determining to what extent the covalently attached phallotoxin was able to exhibit the stabilizing effect. The results of these experiments are shown in Table I. In the control sample, one equivalent of free phalloidin reduced ATP hydrolysis to ca. 7% of the value assessed for normal F-actin. The corresponding activity of the Cys-374 derivative was 12%. Considering that due to the presence of the linker moiety the affinity of the phalloidin derivative to actin was only 87% compared to phalloidin, the stabilizing effect is comparable to that of free phalloidin. In accordance with this high activity, we were unable to obtain a further increase in stabilization by introducing a 10-atom spacer moiety between the toxin and the protein: introduction of a succinoylhexamethylenediamido moiety resulted in a slight decrease of the stabilization effect (17% residual ATPase activity; data not shown).

Table I

Relative steady-state ATPase activity of F-actin substituted at Cys-374 or Cys-10 with the phalloidin derivative, relative to control actin

Modification	Relative ATPase activity
None, without phalloidin	1.00
None, 1 eq. of phalloidin	0.07 ± 0.03
^{374}Cys-S-S-$(CH_2)_2$-CO-NH-PHD	0.12 ± 0.06
^{10}Cys-S-S-$(CH_2)_2$-CO-NH-PHD	2.70 ± 0.7

Control actin steady-state ATPase activity = 0.9 mol $^{32}P_i$ per h per mol of actin at 37°C, equal to 1 0. Mean values (n = 3) are given with S.D.

The high stabilizing capacity of phalloidin attached to Cys-374 of actin shows that the toxin will find its binding position in the filament. Unexpectedly, the sequence of reactions could not be reversed. We examined whether the phalloidin reagent, after complexation to F-actin, would be able to undergo the disulfide exchange reaction with the thiol of Cys-374. This thiol group is the only one exposed in F-actin, and, according to the above results, should be in close vicinity to the reagent. However, no increase in absorbance at 412 nm was observed after 3 h, indicating that this reaction did not occur. On the other hand, the thiol of the Cys-374 moiety of the F-actin stabilized with the phalloidin reagent was still accessible to other thiol reagents added, for example, ODNB [16]. Addition of one equivalent of this reagent resulted in a 50% modification of Cys-374 after ca. 11 min.

The stabilizing effect documented for the covalently bound phalloidin was completely lost when the phalloidin reagent was covalently attached to Cys-10. Here, we measured a steady-state ATPase activity that was enhanced by a factor 2–3, indicating that the peptide in this position has not only lost its stabilizing activity but obviously disturbs one of the actin–actin interfaces or induces a conformational change in the protein that is unfavourable for filament formation.

4. DISCUSSION

According to the atomic model of actin as elucidated by the X-ray analysis of the DNase I complex [7], both the N-terminus and the C-terminus of the protein are situated in subdomain 1. In the same subdomain we find the three amino acid residues which were shown to react with a photoactivatable phalloidin derivative [6], Glu-116 and Met-119 in the middle of a long α-helix and Met- 355 at the end of another short α-helix. All three amino acid residues are located a short distance from the C- terminus. From this spatial arrangement one would expect that a phalloidin derivative, when covalently linked to the penultimate amino acid, Cys-374, must come close to its binding site and should be able to exert the known stabilizing effect. By measuring the steady-state ATPase of such as phalloidin–actin conjugate, we were able to show that this is indeed the case. The amount of stabilization achieved with phalloidin attached to position 374 was nearly equivalent to that obtained with free phalloidin. No stabilizing activity was observed with phalloidin attached to Cys-10. As concluded from the X-ray structure, this point of attachment is in the same subdomain as Cys-374, but opposite to the presumed phalloidin binding site. The thiol group of Cys- 10 is part of a β-sheet and normally not accessible to the solvent. In a previous study we showed that, by a very slow conformational transition in ADP–G-actin, this thiol group becomes exposed to thiol reagents. The addition of ATP and subsequent polymerization fully reverses this conformational change [9]. It is remarkable that introduction of a group having the size of phalloidin together with its linker (ca. 1,000 Da), does not so greatly disturb the architecture of the actin molecule that polymerization becomes impossible at all. While the peptide substituent in this position destabilizes the filaments, it appears that the toxin is still active and able to form crosslinks to other filaments, an effect that might explain the strong gelification observed. It has been documented by NMR studies using fluorine reagents introduced at Cys-374 that the C-terminus of actin has a high degree of mobility [21]. We assume that such mobility of the C-terminus is required for fitting of the covalently bound toxin into the binding site. There is evidence that due to binding of the toxin the C-terminus is shifted to a position different from that taken in normal F-actin. This must be concluded from the observation that a phalloidin reagent added to F-actin will lead to rapid complexation, but not to subsequent formation of the disulfide linkage between protein and toxin. We can exclude the possibility that by binding of the phalloidin reagent the thiol group of Cys-374 is buried since there is still reactivity when another thiol reagent is added. The most likely explanation for the failure, therefore, is that the reaction cannot occur because none of the two reactants can move. Obviously, one effect of phalloidin binding to F-actin is the immobilization of the C-terminus.

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