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ORIGINAL PAPER

The effect of toxins on inorganic phosphate release during actin polymerization

Andrea Vig · Róbert Ohmacht · Éva Jámbor · Beáta Bugyi · Miklós Nyitrai · Gábor Hild

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Abstract During the polymerization of actin, hydrolysis of bound ATP occurs in two consecutive steps: chemical cleavage of the high-energy nucleotide and slow release of the γ-phosphate. In this study the effect of phalloidin and jasplakinolide on the kinetics of P_i release was monitored during the formation of actin filaments. An enzyme-linked assay based spectrophotometric technique was used to follow the liberation of inorganic phosphate. It was verified that jasplakinolide reduced the P_i release in the same way as phalloidin. It was not possible to demonstrate long-range allosteric effects of the toxins by release of P_i from F-actin. The products of ATP hydrolysis were released by denaturation of the actin filaments. HPLC analysis of the samples revealed that the ATP in the toxin-bound region was completely hydrolysed into ADP and P_i. The effect of both toxins can be sufficiently explained by local and mechanical blockade of P_i dissociation.

Keywords Actin · Phalloidin · Jasplakinolide · Inorganic phosphate · Kinetics

Introduction

Actin is a highly conserved 42-kDa protein (Elzinga et al. 1973) in eukaryotic cells. It is important in many cellular processes (e.g. cell division, motility, signalling, chromatin remodelling) (Akiyama and Kawasaki 2006; Gieni and Hendzel 2009; Pollard et al. 2000; Pollard and Borisy 2003). In cells it occurs as monomeric (globular or G-actin) and polymeric (filamentous or F-actin) forms. The globular form of actin can be divided into two large domains. Between these two domains there is a central cleft that contains a bound nucleotide and a divalent cation (Estes et al. 1992) (Fig. 1).

Actin monomers are integrated into a right-handed helix in F-actin by a polymerization process (Holmes et al. 1990). Actin monomers can assemble into filaments after a change in the physicochemical properties of the local environment (e.g. an increase of the ionic strength) and as a result of the effect of some actin-binding proteins (e.g. ARP2/3 complex; formins) (Harris and Higgs 2004, 2006; Mahaffy and Pollard 2006). Polymerization is accompanied by stoichiometric hydrolysis of the actin-bound ATP. Korn et al. (1987) showed for skeletal muscle actin that addition of the subunits and the ATP hydrolysis in a rapidly polymerizing filament had different kinetics. Hydrolysis becomes uncoupled from assembly of the monomers and lags behind (Korn et al. 1987). The measured rate constant for ATP hydrolysis is 0.02 s^{-1} (Pollard and Weeds 1984). ATP hydrolysis results in an ADP.P_i bound protomer within the actin filament. Release of the inorganic phosphate lags behind the hydrolysis with a rate constant of 0.006 s^{-1} (Carlier 1987).

Phalloidin, which is a cyclic peptide from *Amanita phalloides,* can bind tightly to F-actin ($K_{eq} = 36$ nM) (Faulstich et al. 1977; Miyamoto et al. 1986). It stabilizes the structure of F-actin by reducing the dissociation rate of the monomers from the actin filaments (Dancker et al. 1975; Estes et al. 1981; Visegrady et al. 2004). Jasplakinolide is another cyclic peptide, extracted from a marine

A. Vig · B. Bugyi · M. Nyitrai · G. Hild (✉)
Department of Biophysics, Faculty of Medicine, University of Pécs, Szigeti str. 12, 7624 Pécs, Hungary
e-mail: gabor.hild@aok.pte.hu

R. Ohmacht · É. Jámbor
Department of Biochemistry and Medical Chemistry, Faculty of Medicine, University of Pécs, Szigeti str. 12, 7624 Pécs, Hungary

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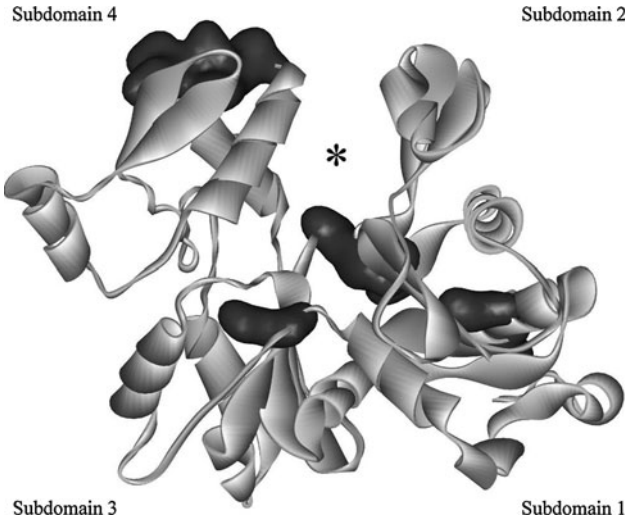

Fig. 1 The solid ribbon model of monomeric actin with bound ATP (PDB ID: 1NWK) (Kabsch et al. 1990). The central cleft between the two large domains is indicated by an *asterisk*. Suggested binding sites for phalloidin are represented by the *black solid* areas (Oda et al. 2005; Vandekerckhove et al. 1985)

sponge (*Jaspis johnstoni*). It also binds F-actin with high affinity ($K_{eq} = 15$ nM) (Bubb et al. 1994) and stabilizes its structure (Visegrady et al. 2004). These two toxins bind the filamentous actin competitively (Bubb et al. 1994). The binding sites for phalloidin have been identified by several research groups (Lorenz et al. 1993; Oda et al. 2005; Steinmetz et al. 1998; Vandekerckhove et al. 1985) (Fig. 1). Both phalloidin and jasplakinolide binding can have local, short-range, or long-range allosteric effects on F-actin (Chik and Schriemer 2003; Orban et al. 2008; Visegrady et al. 2005). Visegrady and his colleagues demonstrated that binding of these toxins to the actin filament cooperatively affected the thermodynamic stability of F-actin. Phalloidin perturbs seven neighbouring protomers whereas jasplakinolide has a stronger effect because it stabilizes 15 neighbouring protomers within F-actin with 1:1 binding stoichiometry (Visegrady et al. 2005). Interestingly, the stabilizing effect of phalloidin was not cooperative on the ADP.BeF_x-actin and ADP.AlF_4-actin filaments, which mimic the ADP.P_i state of F-actin (Orban et al. 2008). It has been suggested that although ATP hydrolysis was not affected, P_i release was inhibited in the presence of phalloidin (Dancker and Hess 1990; Pinaev et al. 1995). The effect of the jasplakinolide on the release of the inorganic phosphate is unknown.

The objective of this work was to study how the binding of phalloidin and jasplakinolide modified the kinetics of release of inorganic phosphate from the newly created actin filaments. The question of whether jasplakinolide has the same effect as phalloidin on P_i release was inspired by their overlapping functional activity (Bubb et al. 1994). We also examined whether the toxins induced only local changes, or whether these effects extended along the actin filament.

It was found that phalloidin and jasplakinolide effectively modified the release of phosphate from newly formed F-actin. The amount of phosphate released decreased linearly with increasing concentration of the toxins whereas the rate of release of phosphate from the unaffected sites of the filament was not affected. The products of ATP hydrolysis were identified after denaturation of the actin filaments. HPLC analysis of the samples showed that breakdown of the ATP was complete even in the toxin-bound region of the actin filament. It was possible to demonstrate that both toxins had only a local, steric blocking effect on P_i release from the actin filament. A possible explanation of the previously seen toxin-induced cooperative effect on F-actin is that release of P_i is required for manifestation of the long-range allosteric effect of the toxin molecules.

Materials and methods

Chemicals

KCl, $MgCl_2$, $CaCl_2$, TRIS (tris-(hydroxymethyl)aminomethane), EGTA, and phalloidin were purchased from Sigma–Aldrich (Budapest, Hungary). ATP and β-mercaptoethanol were obtained from Merck (Budapest, Hungary). Jasplakinolide and the EnzChek phosphate assay kit were obtained from Molecular Probes–Invitrogen (Carlsbad, CA, USA). For the HPLC measurements all reagents were analytical-grade. Freshly bidistilled water was used for preparation of aqueous solutions. Methanol (gradient-grade) and phosphoric acid were purchased from Merck (Darmstadt, Germany).

Actin preparation

Acetone-dried muscle powder was extracted from skeletal rabbit muscle as described by Feuer et al. (1948). The Ca-bound G-actin was prepared by the method of Spudich and Watt (1971) and stored in buffer-A (4 mM Tris–HCl, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM MEA, and 0.005% NaN_3 at pH 7.5). The concentration of Ca-G-actin was calculated by using the absorption coefficient of 0.63 mg^{-1} ml cm^{-1} at 290 nm (Houk and Ue 1974) and with the relative mass of 42 kDa (Elzinga et al. 1973). All measurements were completed on Mg-G-actin. The Ca-G-actin was converted to Mg-G-actin by incubating the sample in the presence of 0.1 mM $MgCl_2$ and 0.2 mM EGTA for at least 5 min (Strzelecka-Golaszewska et al. 1993).

Preparation and use of pyrene labelled actin

Sample preparation

Actin was labelled with pyrene on Cys374 according to the procedure of Criddle et al. (1985). F-actin (1 mg/ml) was labelled for 18 h at room temperature in the presence of a 1.1-fold excess of the fluorescent dye. Labelling was completed by centrifugation at 328,000*g* for 45 min at 4°C. The pellet was suspended and homogenized in buffer-A. After exhaustive dialysis of the sample the pyrene concentration was determined by using the absorption coefficient of 22,000 M^{-1} cm^{-1} at 344 nm. The labelling ratio was typically 90%.

Polymerization assay

Pyrene actin was used to monitor the time dependence of the formation of actin filaments. In these experiments 5% of the total actin was labelled with pyrene. After cation exchange polymerization was started with 100 mM KCl and 2 mM $MgCl_2$. When toxins were used they were added to the sample immediately before addition of the polymerization salts. The actin concentration and the concentration of the toxins were 20 µM. The excitation and emission wavelengths were 365 and 407 nm, with 5 nm slits in both optical paths. The increase of the fluorescence was followed for at least 800 s. The measurements were carried out with a Perkin–Elmer LS50B spectrofluorimeter.

Measurement of P_i release with the EnzChek phosphate assay kit (Webb 1992)

In the presence of P_i, the substrate 2-amino-6-mercapto-7-methylpurine riboside (MESG) is converted enzymatically by the purine nucleoside phosphorylase (PNP) into ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine. This conversion of MESG causes a shift in the position of the substrate's maximum absorbance from 330 to 360 nm.

During the measurements the change of the absorbance at 360 nm was followed for a maximum of 3,000 s at 22°C with time resolution of 1 s. After cation exchange, polymerization of the Mg-G-actin was started with 2 mM $MgCl_2$ and 100 mM KCl in the presence of 20 µM actin, and the kinetic of P_i release was followed with the EnzChek phosphate assay kit. The kit was also used to prepare a calibration curve.

HPLC analysis

HPLC was performed with a Dionex (Sunnyvale, CA, USA) P680 gradient pump, a helium degassing system, a Rheodyne 8125 injection valve with a 20-µl loop (Rheodyne Europe, Bensheim, Germany), and a Dionex 340D UV–visible diode-array detector. A 250 mm × 4.6 mm column packed with 6 µm particle size C_{18} 120 Å reversed-phase material was used for the separations (Szabo et al. 2005). Chromeleon data-management software (Version 6.60 SP3 Build 1485; Dionex) was used to control the equipment and for data evaluation. A multi-step gradient method was applied by using 2.5% methanol–50 mM phosphate buffer (pH 6.0) as solvent A and 70% methanol–50 mM phosphate buffer (pH 6.0) as solvent B at a flow rate of 1.5 ml/min. The gradient profile was: 0.0–7.0 min, from 0 to 28% B; 7.0-9.0 min, 28% B; 9.5 min, 0% B. Chromatographic separations were monitored at 260 nm, where adenosine phosphates has an absorption peak. Twenty microlitres of the samples were injected and the separations were carried out at ambient temperature (Szabo et al. 2005).

Sample preparation for HPLC analysis

G-actin stored in buffer-A or Mg–F-actin polymerized with 2 mM $MgCl_2$ and 100 mM KCl for 3 min in the presence or absence of the toxins were precipitated with 0.6 M perchloric acid. To remove the precipitate the samples were centrifuged for 5 min at 16,100*g*. A proportional amount of K_2CO_3 was added to the supernatant to neutralize the perchloric acid generated acidity of the sample solution. The potassium perchlorate was removed by centrifugation at 16,100*g* for 5 min (van Doorn et al. 1989). The nucleotide content of the supernatant was analysed by HPLC.

Theoretical considerations

The cooperative behaviour of toxin binding to the F-actin has previously been described by Visegrady et al. (2005). Considering that the effect of the toxin on both sides of the bound protomers is equal, the number of the affected protomers is $2k$ where k is the number of the disturbed units on one side of the toxin-bound protomer. The probability that one protomer has $2k$ neighbouring protomers with no bound toxin can be defined as $(1-p)^{2k}$ where p is the probability that a protomer binds the toxin. The probability that a protomer is not affected by any of the toxins is N/N_0, where N is the number of actin protomers in a filament not affected by toxins and N_0 is the total number of protomers in a filament. On the basis of these considerations the following formula can be used to characterise the size of the part of the F-actin affected by toxin binding:

$$\frac{N}{N_0} = (1-p)^{2k+1} \quad (1)$$

In our case the amount of phosphate liberated was regarded as directly proportional to the number of hydrolysing actin protomers. In our situation N is the calculated amount of inorganic phosphate released from the protomers not affected by the toxins, and N_0 is the total amount of released P_i without any toxin bound to the F-actin. The cooperative unit $(2k + 1)$ can be calculated by fitting Eq. 1 to the data points.

Results and discussion

P_i release from the newly formed actin filaments in the absence of toxins

The objective of this study was to reveal the molecular changes behind the stabilizing effect of phalloidin and jasplakinolide on the actin filaments. Release of inorganic phosphate was investigated after actin hydrolysed the bound ATP. Nucleotide exchange on F-actin was blocked by phalloidin whereas nucleotide hydrolysis was left unaffected (Barden et al. 1987). These measurements could not reveal details about what happened to the inorganic phosphate during the polymerization process.

At the beginning of the experiments release of the inorganic phosphate from the newly formed F-actin was monitored in the absence of toxins. As for skeletal muscle actin, the rate of ATP hydrolysis is faster (0.02 s^{-1}) (Pollard and Weeds 1984) than that of P_i release (0.006 s^{-1}) (Carlier 1987), at the beginning of the polymerization P_i release is not affected by the rate of association of the actin monomers. Polymerization tests on pyrene-labelled actin revealed that under the applied conditions at least 90% of the actin monomers were incorporated into the filaments within 180 s after polymerization was started (Fig. 2).

The EnzChek phosphate assay kit is based on the procedure originally documented by Webb (1992). This method is fast enough to follow in real-time the kinetics of P_i release and also gives information about the total amount of product released from the enzymatically cleaved high-energy adenosine-derived nucleotide. The increase of the absorbance at 360 nm was followed at 22°C for at least 1,500 s in the presence of 20 μM Mg-bound actin (Fig. 3). The absorption transients were fitted with the combination of a single exponential function and a linear function. The reason for using the single exponential function was that P_i release from the F-actin followed first-order kinetics (Melki et al. 1996) whereas the linear function was involved because of the slow steady-state ATP-hydrolysing activity of the filaments. Analysis of the data showed that the P_i release rate (k_P) was $3.5 \pm 0.4 \times 10^{-3}$ s^{-1} for the actin in the absence of the toxins. The total amount of P_i released was determined by using a calibration curve

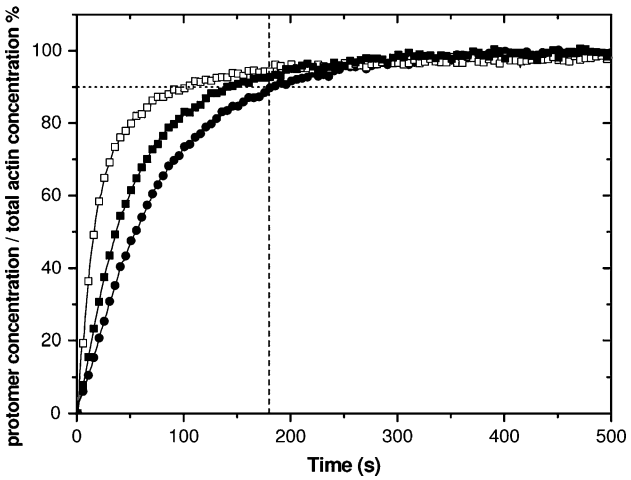

Fig. 2 Polymerization curves of 20 μM actin (5% pyrene-labelled) in the absence of toxins (*filled circles*) and in the presence of 20 μM phalloidin (*open squares*) or jasplakinolide (*filled squares*). The *vertical dashed line* represents 180 s on the x axis and the *horizontal dotted line* corresponds to 90% of the protomer concentration over total actin concentration

acquired with a KH_2PO_4 solution as a source for inorganic phosphate. The amount of phosphate released during the fast phosphate release phase was 20.4 ± 1.5 μM, which indicates that all the actin monomers are incorporated into the filaments and all the phosphates were released and detected by the spectrophotometric method.

The effect of phalloidin and jasplakinolide on P_i release

The P_i-release measurements were repeated in the presence of phalloidin or jasplakinolide at 0, 25, 50, 75, and 100% with the actin concentration kept at 20 μM (Fig. 3). Because of the strong affinity of binding of these toxins to actin ($K_D = 36$ nM for phalloidin (Faulstich et al. 1977) and $K_D = 15$ nM for jasplakinolide (Bubb et al. 1994)), more than 99% of the added toxin bound to the F-actin even at the smallest toxin concentration (5 μM). The polymerization tests completed in the presence of saturating phalloidin and jasplakinolide concentrations showed a small increase in the polymerization rate of actin in the presence of any of the toxins, probably because of their nucleating activity (Mahaffy and Pollard 2008) (Fig. 2).

The release rate at different toxin-to-actin ratios remained nearly the same in the presence of phalloidin ($3.8 \pm 0.3 \times 10^{-3}$ s^{-1}) and jasplakinolide ($3.90 \pm 0.5 \times 10^{-3}$ s^{-1}) as it was for the toxin-free actin filaments (Figs. 3, 4). The amplitude of P_i release decreased gradually as the relative amount of the applied toxins in the solution increased (Figs. 3, 4). At 1:1 molar ratio the maximum effect of the toxins was observed for both of them as no P_i release was detected at these high toxin levels. These results demonstrated that not only the

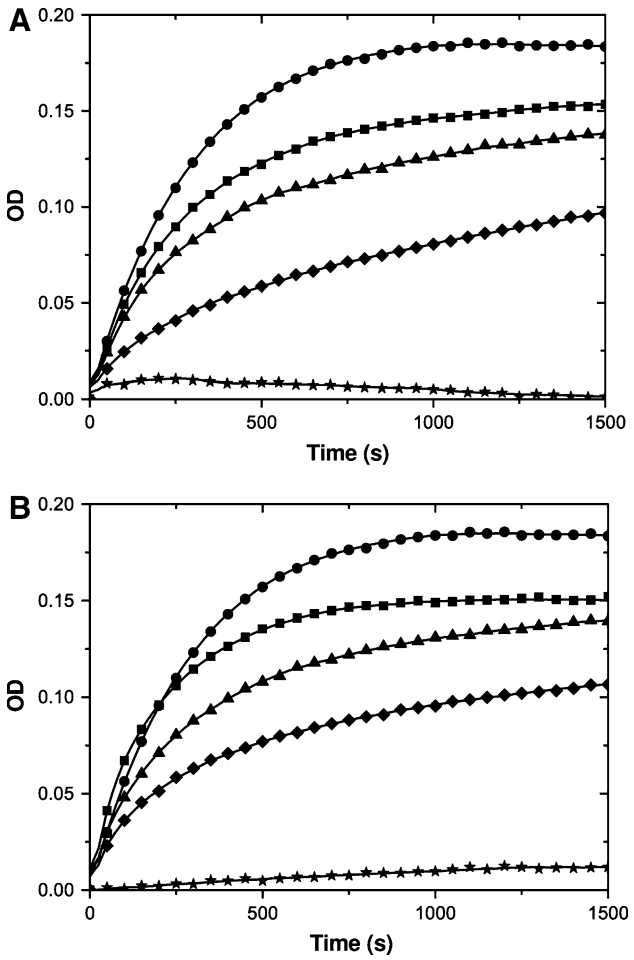

Fig. 3 Change of the spectrophotometric signal with time for phalloidin (**a**) and jasplakinolide (**b**). The amounts of the toxins were: 0% (*filled circles*), 25% (*filled squares*), 50% (*filled triangles*), 75% (*filled diamonds*) and 100% (*filled stars*)

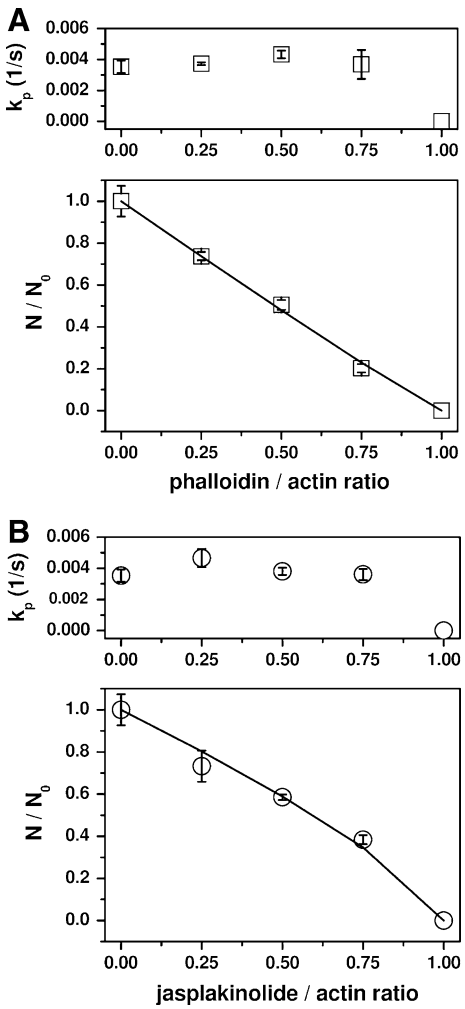

Fig. 4 Plot of calculated N/N_0 values against various toxin-to-actin ratios for phalloidin (**a**) and jasplakinolide (**b**). The *upper parts* of the panels display the measured release rate (k_p) at different toxin-to-actin ratios. The *error bars* represent the calculated standard deviations

phalloidin but also the jasplakinolide effectively blocked P_i release from the toxin-bound parts of the F-actin. Both toxins affected the P_i release similarly, which supports the assumption that, because of the high extent of overlap of the binding sites of the applied toxins, their effect on P_i release is very similar.

The plot of calculated N/N_0 values against toxin-to-actin ratios was analyzed by using Eq. 1 (Fig. 4). The data fits gave k values of 0.03 ± 0.02 for phalloidin and -0.12 ± 0.03 for jasplakinolide (Fig. 4). The size of the cooperative protein unit ($2k + 1$) that could be calculated by use of the revealed k values was approximately 1 for both phalloidin and jasplakinolide. This result indicates that the binding of phalloidin or jasplakinolide affects only the bound protomers within the actin filaments and does not have long-range effects extending to the neighbouring part of the filament around the binding site of the toxins.

Uncovering the state of the nucleotides in the presence of toxins

The results of the kinetic experiments showed that both phalloidin and jasplakinolide reduced the amount of phosphate released from the F-actin. These measurements did not reveal whether ATP hydrolysis and/or P_i release were affected by the toxins. To address this question the products from ATP hydrolysis were identified after being released from the denatured actin filaments. Samples from ATP decomposition were analysed qualitatively and quantitatively by HPLC by using the calibration curves for ATP, ADP, and AMP (Fig. 5). Control measurements with buffer-A showed that the buffer contained mainly ATP and a small amount of ADP without the presence of AMP. The nucleotide content of the 40 μM G-actin sample showed the presence of much larger amounts of ADP and AMP

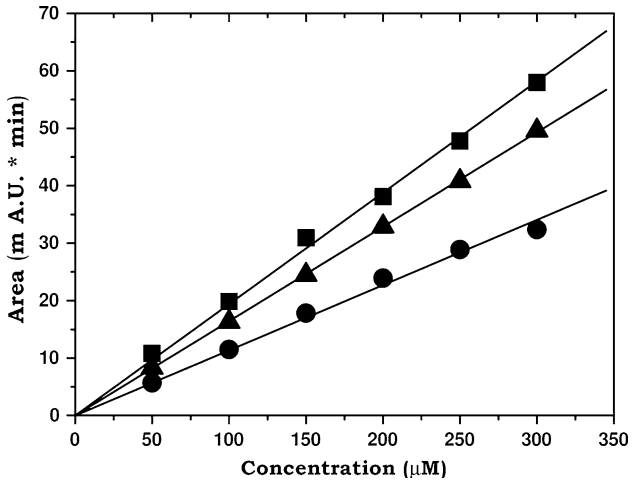

Fig. 5 HPLC calibration curves for the nucleotides. Peak areas for the different nucleotides are presented as functions of ATP (*filled squares*), ADP (*filled triangles*) and AMP (*filled circles*) concentrations

compared with the buffer solution, probably because of the low-level of ATP hydrolysis by the actin monomers (Schuler 2001). The F-actin sample (40 μM) after polymerization for 3 min contained significantly less ATP and more ADP, because of more pronounced ATP hydrolysis by the actin filaments (Table 1) (Pantaloni et al. 1985; Pollard and Weeds 1984). When phalloidin was present during the polymerization process similar decrease in the ATP and increase in the ADP concentration was detected, a result similar to that for the toxin free F-actin sample (Table 1). The results indicate that the same amount of ATP was hydrolysed within unit time in the presence of the phalloidin molecule, as previously reported by Dancker and Hess (1990). The results obtained in the presence of jasplakinolide are similar (Table 1), as predicted from structural studies (Visegrady et al. 2004).

It is reasonable to conclude that the ATP is hydrolysed in the presence of the toxins but the release of the inorganic phosphate is sterically blocked by the bound phalloidin and jasplakinolide, probably in a very similar way.

Conclusions

The results showed that both toxins affected P_i release similarly. Although the first order rate of phosphate release was unchanged, the amount of phosphate released decreased in proportion to the increased concentration of the toxin. Further analysis of the data revealed no signs of long-range allosteric effects of the toxins on P_i release from the F-actin, which suggests that the effect of the toxins was focused only in the near vicinity of their binding region. Denaturation of the proteins made it possible to identify the otherwise unreleased hydrolysis products. In the presence of the toxins hydrolysis of the actin-bound ATP was complete, indicating that only the release of the inorganic phosphate in the ATPase cycle of actin was inhibited by the toxin molecules. The amount of phosphate released decreased in direct proportion to binding of the toxins, which indicates that dissociation of the products after ATP hydrolysis was probably reduced by toxin-induced conformational changes within the actin filament. The absence of allosteric effects suggests that the effects of binding of both toxins can be very adequately explained by local mechanical blockade of P_i release from the nucleotide binding cleft on actin.

From previous studies we know that F-actin built from ATP-bound monomers is more stable and acts like a stiff rod, whereas filaments obtained from ADP monomers only are more flexible (Janmey et al. 1990). After the ATP is hydrolysed P_i dissociation is accompanied by destabilisation of the actin filaments because of loosening the interactions between the protomers (Combeau and Carlier 1988; Muhlrad et al. 1994). The structural dynamics of actin is highly dependent on hydrolysis of the ATP and release of the P_i from the filament. Previous results also showed that phalloidin and jasplakinolide efficiently reduced the flexibility of the F-actin even at a distant point from their binding region (Vig et al. 2009; Visegrady et al. 2004, 2005). A recent study by Orban and colleagues demonstrated that the effect of phalloidin binding was not cooperative when the ADP.P_i state of the actin protomers within the filament was mimicked by ADP.BeF_x or ADP.AlF_4

Table 1 Amounts of the different nucleotides in different samples based on the HPLC calibration curves

	ATP (μM)	ADP (μM)	AMP (μM)	Σ (μM)
Buffer A	162.1 ± 6.4	15.1 ± 4.7	0	177.1 ± 1.8
G-actin	165.8 ± 19.2	27.5 ± 10.3	5.7 ± 0.1	199.1 ± 29.6
F-actin	113.6 ± 0.3	87.1 ± 0.8	7.8 ± 0.7	208.4 ± 0.3
F-actin + phalloidin	98.3 ± 2.1	96.8 ± 6.6	8.7 ± 0.2	203.9 ± 4.8
F-actin + jasplakinolide	100.5 ± 15.8	84.1 ± 8.8	5.7 ± 0.2	188.8 ± 4.5

The presented data are averages from four independent measurements with the calculated standard deviations. The actin concentration was 40 μM. In the presence of toxins the toxin-to-actin ratio was 1:1

molecules (Orban et al. 2008). Based on the non-cooperative effect of the toxins on P_i release it is reasonable to assume that the allosteric effect of the toxins can only be manifested after release of the γ-P_i molecule. From our results we can conclude that in the presence of phalloidin and jasplakinolide the stabilization of the F-actin is probably the result of two separate processes. One is the direct link established between the protomers through the bound toxin molecules, and the other is related to the presence of phosphate trapped within the actin filament.

Understanding the biological relevance of the binding and hydrolysis of ATP by actin is a long standing objective of the actin research field. Our current observations provided further evidence related to the importance of the relationship between the ATP hydrolysis and the accompanying conformational changes within the actin filament. All these results support that the fine tuning of the conformational states of F-actin can be effectively accessed through the hydrolysis products of the bound ATP molecule processed by the actin protomers.

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