The effect on actin ATPase of phalloidin and tetramethylrhodamine phalloidin

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Received 13 June 1995

Abstract Actin polymerization has been studied in the absence of excess nucleotide. Using G-actin ATP monomers, it was shown that mechanical shearing stimulates ATP hydrolysis. The procedures used enabled the detection of differential effects of phalloidin and tetramethylrhodamine-phalloidin, on the P_i-release step of the actin ATPase. It is concluded that tetramethylrhodamine, in contrast to phalloidin, accelerates P_i-release from actin filaments.

Key words: Actin polymerization; ATP hydrolysis; P_i-release; Phalloidin; Rhodamine-phalloidin and mechanical forces

1. Introduction

Crystal structures of actin, hexokinase and HSC70 cognate protein defined a new class of ATPases [1]. In the case of actin, the physiological role of the actin nucleotide hydrolysis is still unclear. With the monomeric form of the protein, G-actin, containing one molecule of tightly bound ATP per actin monomer, hydrolysis of ATP is a slow process [2], but it can be enhanced under certain conditions [3–5], such as sonication. The ATPase activity is primarily expressed in conjunction with polymerization of the actin into filaments [6,7] and is strongly activated by Mg^{2+} ions.

Observations that under some conditions polymers form well in advance of nucleotide hydrolysis [2,8,9], and that filament formation can occur in the presence of non-hydrolysable ATP analogues [10] show that there is no obligatory coupling between the two processes. However, nucleotide hydrolysis does influence nucleation of polymerization and the stability of the filaments formed. Nucleotide hydrolysis on F-actin could perhaps also play a role in chemo-mechanical transduction in the actomyosin system [3,4,11,12].

Phallotoxins bind to actin [13,14]. As determined with the derivative, rhodamine-phalloidin, the toxins bind to actin filaments with a 1:1 stoichiometry [15]. We have studied the ATPase activity related to actin polymer formation under various conditions in the absence and presence of phalloidin derivatives. In the absence of excess ATP, using G-actin·ATP monomers, Mg^{2+} ions stimulate the formation of an actin intermediate containing ADP·P_i which subsequently is incorporated into polymers. Both phalloidin and rhodamine-phalloidin stimulate ATP hydrolysis and formation of the intermediate. We have confirmed that phalloidin inhibits P_i-release [16] from long actin polymers. The fluorescent phalloidin derivative, however, normally used in visualizing actin filaments in motility assays [17,18], did not inhibit P_i-release.

2. Materials and methods

2.1. Protein preparation

Alpha-actin from rabbit skeletal muscle was prepared from acetone powder by the method of Spudich and Watt [19] and further purified by gel filtration through Sephacryl S-300 (Pharmacia Fine Chemicals) in G-buffer (5 mM Tris-HCl, pH 7.6, 0.2 mM $CaCl_2$, 0.5 mM ATP, 0.5 mM DTT) at +4°C, and the peak fractions containing monomeric α-actin were pooled and stored on ice. The actin concentration of pooled fractions was calculated from their absorbance at 290 nm using an extinction coefficient of 0.63. Protein concentrations were also determined using the Bradford method [20].

To be able to measure ATPase activity of actin, and to avoid possible influences from excess ATP and P_i, the protein solution was filtered through a small column (5 × 5 mm) of Dowex 1-X8 (200–400 mesh) equilibrated with G-buffer without ATP. Removal of excess nucleotide unavoidably leads to a gradual loss of ATP from the actin monomers. Since nucleotide-free actin looses its polymerizability rather rapidly, it was important to use the actin for studies of its ATPase activity as soon as possible after the filtration through Dowex 1-X8. Therefore, actin without excess ATP was used in polymerization experiments within 5 min after the Dowex treatment. The maximal amount of ATP hydrolyzed indicated that 60–80% of the actin was still active at the time of the experiment. Measurements of the amount of protein passing through the filters at the end of the incubations led to the same conclusion.

2.2. Analysis of ATP hydrolysis and P_i-release

Polymerization of actin was induced at room temeprature by adding $MgCl_2$ or KCl (final concentrations 1 mM and 100 mM, respectively) or both salts. Samples were withdrawn at increasing times of incubation and immediately processed for P_i analysis. For the determination of total P_i (protein-bound plus free P_i), 200 μl of the incubation mixtures were added to 200 μl of 0.6 M perchloric acid (PCA) and processed as described below. To determine free P_i plus P_i bound to non-filamentous actin, filaments were removed by filtration of samples through millipore filters (Millipore, cat.no. HAWPO1300, filter type HA; pore size 0.45 μm, diameter 13 mm). For this, 300 μl of the polymerization mixtures were applied to filters and forced through them using gentle suction (underpressure: −100 mmHg). Two hundred microliters (200 μl) of the filtrates were then immediately mixed with 200 μl of 0.6 M PCA. The PCA extracts of unfiltered and filtered samples were centrifuged for 2 min at 5000 rpm to remove protein precipitates. Then, 360 μl of the supernatants were mixed with 360 μl of Malachite green reagent [21]. After 40 min of incubation at 25°C, the absorbance of the reaction mixtures was measured at 650 nm. Samples of G-buffer without ATP was treated in the same manner to provide blanks. The Malachite green reagent was prepared according to Dancker and Hess [16].

2.3. Effect of mechanical forces on G-actin ATPase

It is important to note here that the actin ATPase is easily activated by mechanical forces during the filtration steps used in the above analysis. Too fast passage of actin through filters activates the actin

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SSDI 0014-5793(95)00724-5

ATPase even under non-polymerizing conditions (G-buffer). To avoid undue mechanical activation, columns of Dowex 1-X8 was prepared by sedimenting the resin onto loosely packed glass wool stoppers in plastic syringes, and samples of G-actin were allowed to pass through the Dowex without applying any pressure on the columns. Samples passed through the columns in about 5 min. By the same token, a low under-pressure (−100 mmHg) had to be used in removing filamentous actin by filtration through millipore filters. Low values of P_i obtained with control samples and with samples taken directly after addition of Mg^{2+} indicate success in avoiding mechanical activation of the actin ATPase. Fig. 1 illustrates the hydrolysis of ATP by actin under non-polymerizing conditions in samples that were left undisturbed and in samples that were mechanically disturbed by successive filtrations through millipore filters. Samples of actin were incubated in the absence and presence of excess ATP. At 50 min an equivalent of 3% of the G-actin-bound ATP had been hydrolysed in the absence of excess ATP, assuming no loss of ATP from the actin. Slightly more ATP was hydrolysed in the presence of 20 μM ATP. In samples that were repeatedly forced through Millipore filters increasing amounts of ATP was hydrolysed. After 40 min, during which the samples had been passed through filters 4 times, 25% of the actin-bound ATP was hydrolyzed. In the presence of excess ATP slightly higher ATP consumption was observed.

2.4. Actin polymerization

Polymerization of actin was estimated by recording the decrease in the amount of protein passing through millipore filters (as above). Polymerization of actin (0.5 mg per ml) was also monitored by viscosimetry at 25°C using Cannon–Manning semi-micro viscosimeters requiring 0.7 ml samples and having a flow-time of about 60 s. Ultracentrifugation in a Beckman airfuge was used as a third way to determine the extent of filament assembly. For sedimentation of F-actin samples were spun at 30 psi for 15 min.

2.5. Reagents

Adenosine 5′-triphosphate (disodium salt) and Dowex 1-X8 was obtained from Serva, Heidelberg, Malachite green oxalate from Merk, Darmstad, and phalloidin from Boehringer-Mannheim. Tetramethylrhodamine-phalloidin was purchased from Molecular Probes Inc., Junction City, OR, USA. All other chemicals of reagent grade were purchased from Sigma Chemicals Co., St. Louis, ND or from Merck, Darmstad.

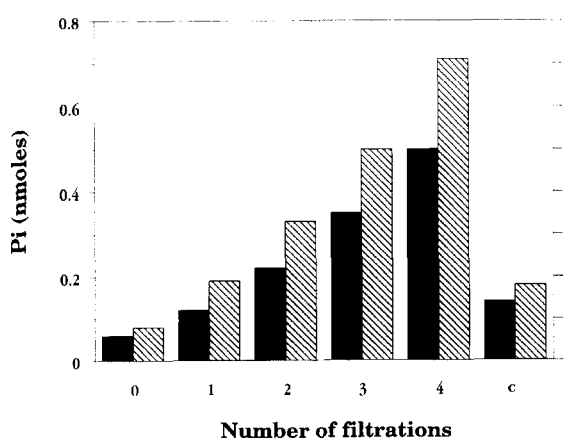

Fig. 1. Effect of mechanical forces on the ATPase activity of G-actin. A G-actin solution (0.5 mg/ml) was passed through a Dowex 1-X8 column to remove free ATP. One part of the solution was then filtered repeatedly through nitrocellulose filters (pore diam. 0.45 μm) under suction and the accumulation of P_i was determined after each filtration (left columns). Twenty (20) micromolar ATP was added to a second part of the G-actin solution which was then treated in the same way and analyzed for P_i-release (right column). A control sample (C) was incubated for 50 min in parallel without filtration.

3. Results

3.1. ATP hydrolysis and P_i-release during polymerization of α-actin

The kinetics of ATP hydrolysis and P_i-release during polymerization of actin induced by the addition of either 100 mM KCl (Ca^{2+} at the high affinity site of actin) or 100 mM KCl, 1 mM $MgCl_2$ (Mg^{2+} partially replacing the Ca^{2+} at the high affinity site with Mg^{2+}) are illustrated in Fig. 2. Samples of α-actin were taken at various times after addition of polymerizing salts and analyzed for total acid soluble P_i and for acid soluble P_i passing through nitrocellulose filters (see section 2).

With 100 mM KCl as polymerizing salt there was a long lag phase in the hydrolysis of ATP. The reaction reached maximal values with $t_{1/2}$ of 35 min. The amount of P_i passing through the filters increased slowly and steadily with time during the period studied. To begin with the latter values exceeded those obtained for total P_i indicating that the filtration of the samples through the nitrocellulose filters had caused some activation of the actin ATPase.

A 20-fold increase in the initial rate of ATP hydrolysis was observed in the presence of Mg^{2+} ions (Fig. 2; open diamonds). Unexpectedly, all of the acid extractable P_i formed passed through the nitrocellulose filters during the first 4 min of the reaction. After reaching a peak value at 4 min, the amount of acid extractable P_i in the flowthrough fraction decreased rapidly to about 50% of the peak value. After that the amount of acid soluble P_i detected in the filtrates increased again, and eventually reached the same maximal values as obtained for total acid soluble P_i.

These results suggested that in the initial phase of the polymerization reaction in the presence of Mg^{2+} (no excess ATP) a filterable intermediate containing ADP·P_i was assembled. The intermediate accumulated at 4 min contained close to 50% of the total hydrolyzable ATP. The equally rapid disappearance of acid soluble ATP from the filtrates reflected the conversion of the intermediate into long filaments. Inorganic phosphate was then released from the F-actin·ADP·P_i at a relatively slow rate to form the end product F-actin·ADP.

That P_i in the filtrate to a large extent was bound to a filtrable form of actin was confirmed by direct analysis of the material on Dowex 1-X8. Inorganic phosphate will be retained by the Dowex resin, whereas protein-bound P_i will pass through unadsorbed. In this case 50% of the acid soluble P_i in the samples from the millipore filtration did not bind to Dowex 1-X8 (data not shown). From the analysis in Fig. 2, it was expected that even more of the P_i would be bound to actin. The most likely reason that this is not the case is that extra time elapsed before the final P_i analysis and/or that the actin in this case was exposed to further mechanical forces.

Analysis of the amount of protein passing through the nitrocellulose filters after increasing times of incubation in the presence of $MgCl_2$ and KCl showed that at 4 min more than 90% of the protein passed through the filters, i.e. was unpolymerized or oligomeric. At 10 min the major part (ca. 75%) and at 60 min >95% of the protein was retained by the nitrocellulose filters, i.e. was in the form of long stable filaments. Viscosimetric analyses confirmed that in 4 min only a minor fraction (<30%) of the actin had been transformed into filaments, but that at 10 min the viscosity of the actin samples had reached 90% of the final level of viscosity. Similar conclusions could be drawn

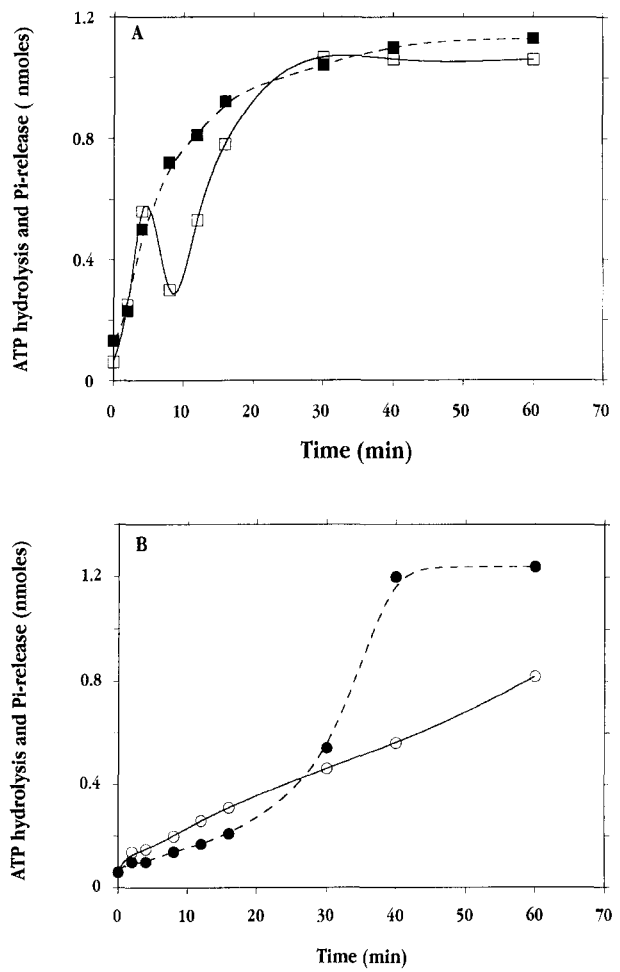

Fig. 2. Hydrolysis of ATP and P_i-release during actin polymerization. Nucleotide-free G-actin was prepared as described in Fig. 1. Polymerization of actin (0.5 mg per ml) was induced by addition of either 100 mM KCl + 1 mM $MgCl_2$ (A) or 100 mM KCl (B). Samples were analyzed for total P_i (closed squares in A; closed circles in B), and 'free' P_i (= P_i passing through nitrocellulose filters; open squares in A; open circles in B).

from the results of analysing polymerization mixtures by ultracentrifugation (airfuge). Primary data from protein analysis are not shown except in Fig. 3.

3.2. *Effect of phalloidin on ATP hydrolysis and P_i-release during polymerization of α-actin*

The effect of phalloidin on ATP hydrolysis and P_i-release by α-actin induced to polymerize by the addition of 1 mM $MgCl_2$ (no KCl added) is shown in Fig. 3. Here, samples taken from the flowthrough from the millipore filtration were also analyzed for protein content using the Bradford method [20]. These analyses indicated that there was a lag phase in the disappearance of protein from the millipore filtrates, after which the amount of protein dropped precipitously, indicating polymerization of the actin. The analyses suggested that about 5% of the actin had polymerized at 2 min of incubation and not more than 20% at 4 min. At these times points, however, 45% and 71%, respectively, of the final level of ATP hydrolysis had already been reached.

The amount of acid soluble P_i in the filtrates followed the total amount P_i formed rather closely during the initial phase of the reaction and reached peak values at about 4 min. During the subsequent 4 min, the amount of acid soluble P_i in the filtrate dropped by about 85% suggesting rapid incorporation of the filtrable actin·ADP·P_i intermediate into F-actin·ADP·P_i. In the third phase of the reaction, there was a gradual reappearance of P_i in the millipore filtrates suggesting P_i-release from the F-actin. At 30 min of incubation, however, about 80% of the total P_i formed was still bound to actin. This experiment confirms the observations made by Dancker and Hess [16] and strengthens the conclusion that a large fraction of the actin is present in an actin·ADP·P_i intermediate already in the early phase of the reaction.

A comparison of the kinetics of ATP hydrolysis and P_i-release by actin in the absence and presence of either phalloidin or rhodamine-phalloidin is shown in Fig. 4. Panel A shows that ATP hydrolysis on actin is accelerated by both phalloidin and its fluorescent derivative, rhodamin-phalloidin, although the effect of rhodamin-phalloidin was not as pronounced as that of phalloidin itself. Panel B illustrates how the amount acid soluble P_i that appears in the filtrates changes with time during polymerization under the different conditions. It is obvious that in the presence of phalloidin, the formation of the actin·ADP·P_i-containing intermediate is accelerated, and that phalloidin inhibits P_i-release from the non-filterable actin filaments that eventually form. It is also clear that rhodamine-phalloidin, like phalloidin, stimulates the appearance of acid soluble P_i in the flowthrough from filtration through millipore filters.

The lag phase in the polymerization of actin in the 1 mM $MgCl_2$ containing buffer used in the experiment of Fig. 4 is longer than that seen in the presence of both $MgCl_2$ and KCl. Therefore, with the choice of time points, the peak in the amount of filterable acid soluble P_i that should have appeared with actin was missed in this particular experiment. The same situation was at hand with the sample containing rhodamine-phalloidin.

4. Discussion

Detailed studies of the kinetics of actin polymerization and

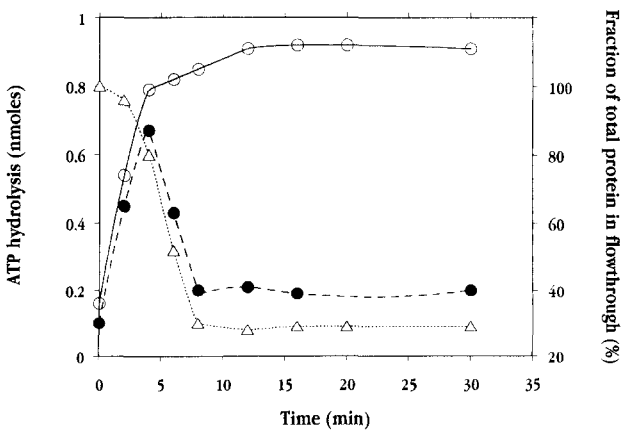

Fig. 3. The effect of phalloidin on ATP hydrolysis and P_i-release during actin polymerization. Nucleotide-free actin (12 μM) was mixed with phalloidin in equimolar amounts (12 nmol). Polymerization was then induced with 1 mM $MgCl_2$. Samples taken at various times of incubation were analyzed for ATP hydrolysis (open circles), P_i-release (filtrate; closed circles), and protein content (open triangles).

nucleotide hydrolysis in vitro [6,7] have indicated that elongation of actin filaments involves the addition of G-actin·ATP monomers to filament ends. Subsequent hydrolysis of the nucleotide takes place in two steps; cleavage of F-actin-bound ATP forming a relatively long-lived and more stable intermediate (F-actin·ADP·P_i), followed by slow release of P_i. Similar rates of elongation are seen with either Mg^{2+} or Ca^{2+} occupying the high-affinity cation binding site on actin. The nucleation reaction, however, is apparently much faster with Mg^{2+} than with Ca^{2+}. These and related observations support the view that Mg^{2+} induced nucleotide hydrolysis gives rise to G-actin·ADP·P_i which facilitates the formation of nuclei containing both actin·ATP and actin·ATP·P_i [6,7,22].

Rhodamine-phalloidin binds to actin filaments assembled in Mg^{2+} and KCl with a 1:1 stoichiometry and a dissociation equilibrium constant of 10–20 nM [15], and phalloidin itself appears to have a 5- to 10-fold higher affinity [23]. Rhodamine phalloidin is commonly used to label actin-containing filaments in cells and extracts of cells, and actin filaments labelled with the fluorescent derivative of phalloidin, tetramethyl rhodamine-phalloidin, can move on tethered myosin in in vitro motility assays [18,24]. It has been proposed that hydrolysis of ATP on filamentous actin might be an essential step in the mechanism of chemo-mechanical transduction in contracting muscle fibers [3,4,11,12]. If tetramethyl rhodamine phalloidin inhibits P_i-release as in the case for phalloidin [16], it would be difficult to sustain this argument. Therefore, it was of interest to test the effect of rhodamine-phalloidin on ATP hydrolysis and P_i-release and compare it with the effect of underivatized phalloidin. Our results indicate clearly that rhodamine-phalloidin, in contrast to phalloidin, neither inhibits ATP hydrolysis nor the release of P_i from F-actin·ADP·P_i formed in its presence.

In the course of these experiments, the actin ATPase proved to be extremely sensitive to shearing forces encountered during filtration. For example, as shown in Fig. 1, there was a direct proportionality between ATP hydrolysis and the number of passages of a given G-actin solution through millipore filters. As described in section 2 this complication can be alleviated by lowering the net pressure used to draw the solution through the filters. The effect of external forces on the actin ATPase is reminiscent of the effect of sonication on solutions containing F-actin. In both cases, the actin ATPase is sensitive to mechanical forces in solution. The effect of sonication on actin filaments was originally interpreted as exchange and hydrolysis of ATP coupled to reversible length changes within the filaments. Myosin has been observed to accelerate the actin ATPase for both monomeric [5] and filamentous actin [4]. These observations support our proposed model of muscle contraction. In this model, the binding of myosin forcibly opens the multiple-domained actin monomers, facilitating nucleotide exchange and hydrolysis on actin filaments. Force is produced by actin during the propagation of contraction waves monomer by monomer along filaments, successively catalyzing the release of P_i from actin.

Our procedure, in which G-actin solutions are passed through Dowex to remove excess ATP, and where the effects of shearing forces on the actin ATPase are minimized, made it possible to investigate the early phase of the polymerization reaction in the absence and presence of phalloidin and its derivative tetramethyl rhodamine-phalloidin. With respect to P_i-release, our results confirm those of Dancker and Hess that

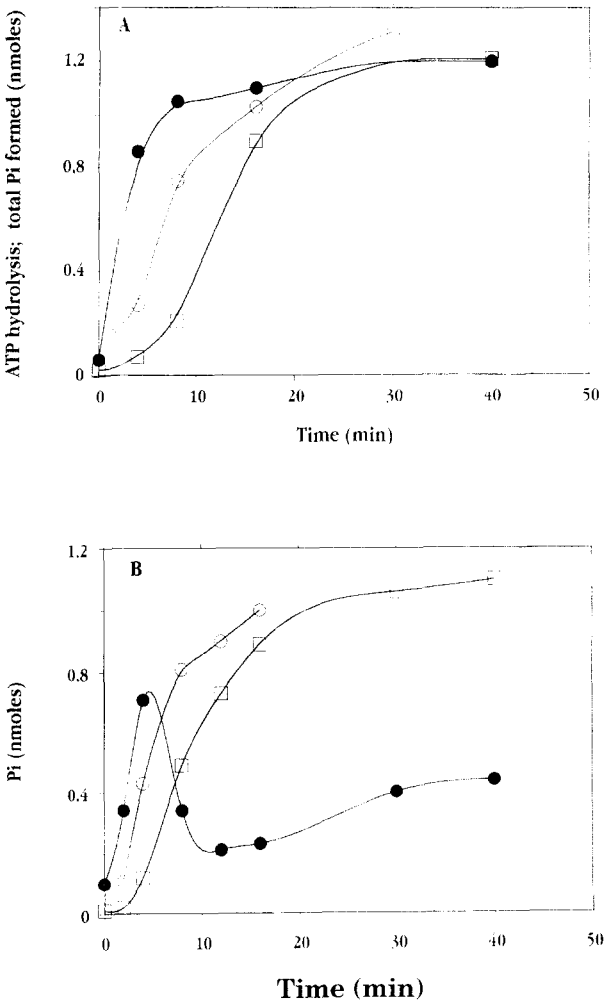

Fig. 4. Kinetics of ATP hydrolysis and P_i-release by actin in the absence and presence of phalloidin or rhodamine-phalloidin. Actin (12 nmol), from which free ATP had been removed by Dowex 1-X8 treatment, was mixed with 10 nmol of phalloidin or rhodamine-phalloidin. Polymerization was induced by the addition of 1 mM $MgCl_2$. Panel A illustrates the ATP hydrolysis measures as total P_i formed in the absence (open squares) and presence of rhodamine-phalloidin (open circles) or phalloidin (closed circles). Panel B illustrates the appearance of 'free' P_i in the flowthrough from the nitrocellulose filters (rhodamine-phalloidin case, open circles; phalloidin case closed circles).

phalloidin itself strongly inhibits the release of P_i, lending stability to the filaments. However, in the case of its derivative tetramethyl rhodamine-phalloidin, P_i-release is not inhibited in the presence of Mg-ATP. Therefore, models of muscle contraction, in which P_i-release from actin is associated with work production, cannot necessarily be ruled out on the basis that a phalloidin derivative is present in in vitro motility assays. Phalloidin and its derivatives are known to alter both the enzymatic and mechanical behaviour of myofibrils from various sources [25–27]. Tetramethylrhodamine-phalloidin is used routinely to visualize actin filament translocation in vitro. Our results indicate that phalloidin derivatives might affect the actomyosin system differently than phalloidin, and that in either case, the chemistry of the actin ATPase cannot be assumed to be unaffected by their presence.

The improved procedures for investigating the actin ATPase, enabled us to distinguish events at early time points in the

polymerization reaction. Fig. 2A and B demonstrate that the rate of elongation, once nucleation has taken place, does not depend on upon the nature of the divalent cation present, whereas Mg^{2+} greatly accelerates the formation of nuclei. Fig. 3 shows that phalloidin increases the intrinsic rate of ATP hydrolysis, whereas P_i-release is inhibited, confirming the results of Dancker and Hess [16].

One reason commonly given for the stabilizing effect of phalloidin on actin filaments is that it inhibits the ATPase activity associated with sonication [28]. In structural terms, this effect of phalloidin is pictured as stabilizing the two-start helical bonds in the original Oosawa model [3]. If P_i-release is assumed to accompany the formation of the two-start bond, stabilizing this bond prevents further cycles of ATP hydrolysis, thereby explaining the effect of sonication on the ATPase. However, the present study suggests that stabilization by phalloidin could also be achieved by stabilizing the ADP·P_i state itself, to the extent that the shearing forces during sonication would be insufficient to overcome the activation barrier to forming the two-start contact. Thus the effect of phalloidin would be to stabilize the F-actin·ADP-P_i metastable state, rather than the F-actin·ADP helical 'ground state'.

Acknowledgements: We gratefully acknowledge grants from the Royal Swedish Academy of Sciences supporting joint research projects between Sweden and the former Soviet Union. Grants to UL from the Swedish Cancer Foundation and the Swedish Natural Science Research Council and to CES from National Institute of Health are also gratefully acknowledged.

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