Chapter 3

The Connection Between Actin ATPase and Polymerization

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Remodeling of the actin filament system in cells results from strictly regulated polymerization and depolymerization of actin, where hydrolysis of actin-bound ATP is crucial. Actin–actin interactions are influenced by the state of the bound nucleotide, and many microfilament regulators influence the actin ATPase by binding preferentially either to ATP/ADP·Pi- or ADP-bound actin. This chapter summarizes observations made concerning the actin ATPase and its role in the biological activity of actin and actin filaments.

I. ACTIN MICROFILAMENT SYSTEM

Actin and myosin, organized into supramolecular structures, cooperate to generate the force necessary for many types of dynamic cellular transport processes. As part of the energy-transducing mechanism in muscle cells, they generate large-scale movements.

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In nonmuscle cells, the actin microfilament system (MFS) drives cell motility, cytokinesis, and vesicular movements. A weave of actin polymers (filaments) found in juxtaposition to the inner surface of the plasma membrane of all cells is intimately coupled to signal transduction controlling the formation of the actin filaments and their involvement in force-generating processes. The organization of the MFS is constantly being remodeled in response to transmembrane signals generated in the interactions between cells and between cells and extracellular matrices or soluble molecules like growth factors and hormones binding to cell surface receptors. All these processes ultimately depend on the hydrolysis of ATP, not only just on myosin but also on actin.

II. ATOMIC STRUCTURE OF THE ACTIN MONOMER

The actin molecule has two major domains, each of which is divided into two subdomains (Kabsch *et al.*, 1990). Subdomains 1 and 3 form a flexible base of the molecule. The purine of the ATP is sandwiched in a hydrophobic pocket between subdomains 3 and 4, and the polyphosphate tail is held by two loops originating from subdomains 1 (P1-loop) and 3 (P2-loop) (Fig. 1). The divalent cation, chelated by the ATP phosphates, makes contacts with residues around the base of the interdomain cleft. The actin monomer can

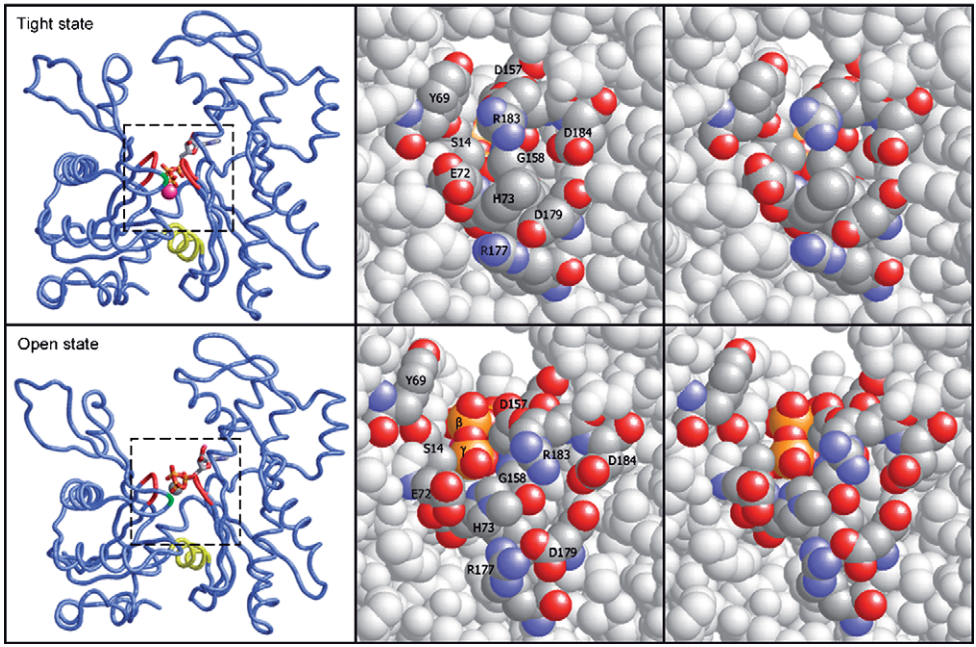

Figure 1. Comparison of the tight and open states of actin in profilin:β-actin crystals. The overview to the left outlines the area shown in stereo containing the barrier residues E72, H73, R177, D179, and R183, the phosphate-binding loop residues S14, D157, and G158, and finally the hydrogen bond-forming residues Y69 and R183. In the open state (bottom row) the β- and γ-phosphates (yellow) of the ATP are exposed. Pdb accession codes 2BTF and 1HLU, respectively. Figure published in *J. Mol. Biol.* (2002) 317, 577–589.

exist in different conformations, depending on the status of the actin-bound ATP, the nature of the divalent cation (Ca^{2+} or Mg^{2+}) at the high-affinity site or at additional sites, and the degree of oligomerization (Moraczewska *et al.*, 1999; Schüler, 2001).

The structures of different actin orthologues cocrystallized with different actin-binding proteins all have a closed nucleotide-binding cleft (Vorobiev *et al.*, 2003), corresponding to the tight state found for β-actin in the profilin:β-actin crystals (Schutt *et al.*, 1993). The conformation of actin appears relatively unchanged regardless of whether the nucleotide is ATP or ADP, or the tightly bound cation is Ca^{2+} or Mg^{2+}. In the light of many observations indicating that the actin can attain different conformations depending on the nature of bound ligands, this may seem contradictory (Schüler, 2001; Strzelecka-Golaszewska, 2001). The relative invariability in most actin crystal structures, however, may be explained by clamping of the two domains by DNase I binding across the cleft between subdomains 2 and 4, or by packing interactions in the case of the gelsolin subfragment 1:α-actin crystals, as discussed previously (Nyman *et al.*, 2002; Sablin *et al.*, 2002). This may also be the case in the crystal structure of tetramethylrhodamine maleimide (TMR)-derivatized α-actin in the ADP state (Otterbein *et al.*, 2001). In this tight state structure, however, subdomain 2 has attained a different structure as compared with previously determined α-actin with a small helical stretch apparently formed through rotation around the same hinge region that is involved in the movement of subdomain 2 in the tight-to-open state transition of profilin:β-actin (Chik *et al.*, 1996). It should be noted that the rotation of subdomain 1 with respect to subdomains 3 and 4, intrinsic to the opening of the nucleotide-binding cleft in profilin:β-actin, is not seen in TMR–actin. It was reported that binding of TMR to actin does not significantly influence DNase I–actin interaction or the susceptibility of the actin to subtilisin cleavage in subdomain 2, which implies that the solution structure of TMR–actin is closely similar to the nonconjugated protein (Kudryashov and Reisler, 2003). Another possibility is that the binding of TMR between subdomains 1 and 3 locks the protein in the tight state. There is evidence for allosteric coupling between the C-terminus and subdomain 2. For instance, there is an increase in the K_{diss} for the profilin–actin interaction after introduction of a P38A mutation (subdomain 2), and reciprocally, replacing cysteine 374 with a serine lowered the affinity of the actin for DNase I binding (subdomain 2) (Aspenström *et al.*, 1993). Likewise, removal of C-terminal residues of actin affected the proteolytic sensitivity of subdomain 2 (Strzelecka-Golaszewska *et al.*, 1993). Figure 2 further illustrates the influence of the C-terminal C374S mutation on the thermal stability of actin in the Mg^{2+}- as well as the Ca^{2+}-bound state as assayed by DNase I inhibition (Schüler *et al.*, 2000a). Clearly, the mechanisms behind the allosteric coupling indicated by these modifications are still unclear.

III. PROFILIN:β-ACTIN CRYSTAL

In the profilin:β-actin crystal, the actin molecules are also bridged across subdomains 2 and 4. Here, a neighboring actin molecule is responsible for the bridging, and the cleft can open and close in response to changes in ionic conditions, despite the bridging (Fig. 3). This is possible through intramolecular hinge and shear movements

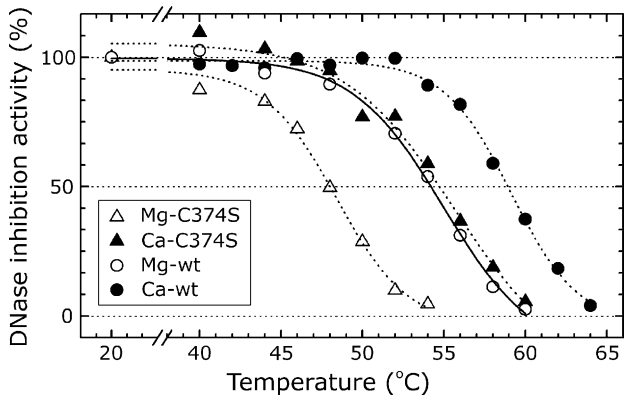

Figure 2. Thermal stability of β-actin carrying the C-terminal mutation C374S. Melting curves of monomeric actins determined with the DNase I inhibition assay as described earlier (Schüler *et al.*, 2000a).

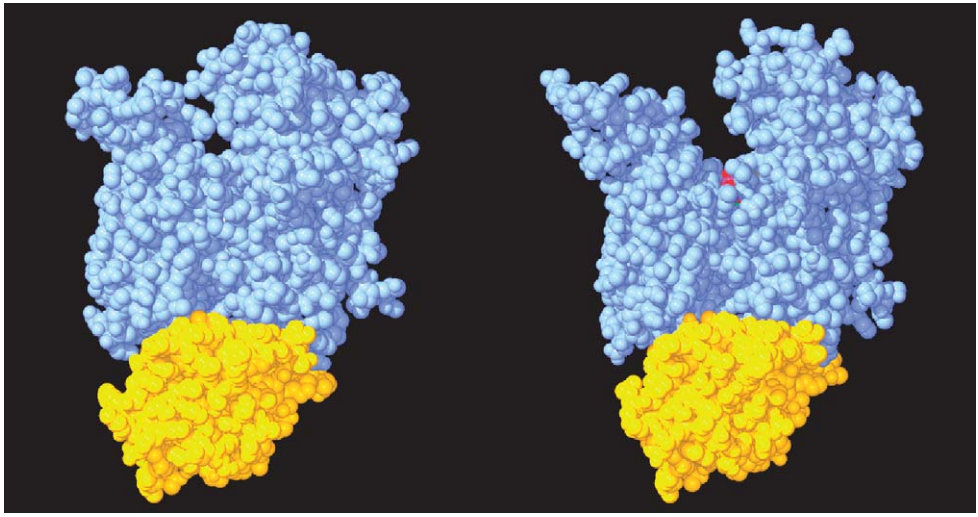

Figure 3. The profilin:β-actin closed and open states (pdb accession codes 2BTF and 1HLU, respectively). Overview of the crystal structures of profilin:β-actin solved in 3.5 M NH_4SO_4 (closed state, left) and 1.8 M potassium phosphate (open state, right) in the presence of Ca^{2+} and ATP, illustrating the magnitude of the conformational difference between the two states. The polyphosphate tail of the ATP is exposed in the open state (oxygen O1beta, red dot). See also Fig. 1.

coordinated between the bound actin molecules (Schutt *et al.*, 1989; Chik *et al.*, 1996; Page *et al.*, 1998). Exchange of ADP and AMP for ATP in the profilin:β-actin crystals significantly influences their diffraction (Schutt *et al.*, 1989). In the tight state structure, the terminal phosphates of the nucleotide are buried (see also Fig. 1, upper

panels), held by the β-hairpin loops, N12-C17 (P1) and D156-V159 (P2), which protrude into the interdomain cleft from subdomains 1 and 3, respectively. The β-phosphate is hydrogen bonded to the amide nitrogens of S14, G15, M16, and D157, and the γ-phosphate is bound to amide nitrogens of S14, D157, G158, and V159. The most dramatic difference between the two states, the opening of the interdomain cleft, results in an outward shift of the N12-C17 loop, exposing the phosphate tail of ATP to solution (Fig. 1, lower panels) (Chik *et al.*, 1996).

In the open state, the hydrogen bond with the amide nitrogen of G15 shifts from the O1 oxygen to the O2 oxygen of the β-phosphate, while the hydrogen bonds of G158 and V159 to the γ-phosphate are broken (Chik *et al.*, 1996). In the tight state, there are two hydrogen bonds that span the nucleotide-binding cleft. These two bridging hydrogen bonds, between MeH73 and the carbonyl oxygen of G158, and between the guanido group of R183 and π-electrons of the ring of Y69 (for bond type see Levitt and Perutz, 1988), stabilize the closed ATP form of the actin molecule. In the open state, these hydrogen bonds are broken.

IV. INTERDOMAIN CONNECTIVITY IN ACTIN

Exchange of ADP for ATP in actin results in a significant reduction in the stability of the protein, and removal of the nucleotide leads to rather rapid loss in polymerizability (Asakura and Oosawa, 1960), demonstrating the importance of the ATP γ-phosphate for holding the two major domains in position. In addition to the loop-phosphate-loop links and the bridging hydrogen bonds discussed earlier, the two major domains of actin are connected through a charge network involving mostly long-chained residues (Fig. 1): E72 and MeH73 from one side of the cleft and D157, R177, D179, and R183 from the other. The presence of a methyl group on the ε2-nitrogen of the imidazole ring of H73 increases the basicity of the δ1-nitrogen, thereby strengthening the hydrogen bond connecting this nitrogen with the carbonyl group of G158. These residues shield the ATP phosphates from the solvent on one side of the molecule. On the opposite side, there is another set of large residues (M16, K18, K336, and Y337) separating the polyphosphate tail from solvent in both the open and tight states. The transition from the tight to open state does not result in any major changes in this barrier region.

Investigations of the effect of mutations in the nucleotide-binding cleft on the spatial relationship between the major domains of actin have shown that the H73A mutation, as well as mutations in the loops binding the phosphates, causes a significant decrease in the affinity for DNase I (Chen and Rubenstein, 1995; Schüler *et al.*, 1999, 2000b; Nyman *et al.*, 2002). The mutations H73A, R177D, S14C, and the double mutation S14C/D157A all destabilized the molecule at increased temperatures, caused increased nucleotide exchange rates, and reduced polymerization rates. Replacing H73 with positively charged residues (arginine or lysine) made the actin more stable, whereas introduction of glutamic acid destabilized the protein (Yao *et al.*, 1999), further illustrating the coordinating position of H73 in the charge network

(Fig. 1) and its importance for the stability and polymerizability of actin (Nyman *et al.*, 2002). See also discussion of MeH73 later.

V. ACTIN ATPASE

A. Monomeric Actin Hydrolyzes ATP

Addition of salts (including Mg^{2+} ions) to a solution of monomeric actin, causing polymerization, increases the rate of ATP hydrolysis approximately by a factor of 100 (Pollard and Weeds, 1984), suggesting a tight coupling between actin–actin interactions and the hydrolysis of ATP. Thus, the slow hydrolysis of ATP in buffers stabilizing the monomeric form of actin has been seen as a consequence of the formation of unstable oligomers of actin in the solution and not as an expression of an *intrinsic* ATPase activity of the monomer (Mozo-Villarias and Ware, 1985; Newman *et al.*, 1985). However, as demonstrated in Fig. 4, the ATPase activity per actin monomer under nonpolymerizing conditions is independent of the total monomer concentration over a broad range of actin concentrations, strongly suggesting that the ATPase activity of actin is independent of oligomerization, that is, monomeric actin has an intrinsic ATPase activity. This activity ($0.6 \pm 0.11\ h^{-1}$) is in the same range as the activities of the heat shock protein Hsc70 or its isolated ATPase domain (Ha and McKay, 1994;

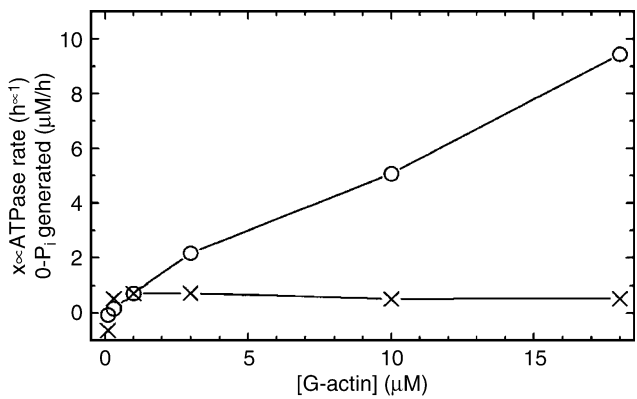

Figure 4. Actin in the monomeric state has ATPase activity. Bovine cytoplasmic β/γ-actin was prepared from calf thymus as described (Lindberg *et al.*, 1988). Monomeric actin in G-ATPase buffer (5 mM tris-HCl pH 7.6, 0.5 mM ATP, 0.67 µM [γ-^{32}P]-ATP (3000 Ci/mmol; Amersham Pharmacia), 0.1 mM $CaCl_2$, 0.5 mM DTT) was converted to Mg-actin by incubation with 0.2 mM EGTA + 50 µM $MgCl_2$ for 15 min at RT. The actin was diluted in the same buffer, including all components listed, to concentrations of 0.1, 0.3, 1.0, 3.0, 10.0, and 18.7 µM and incubated at 25 °C. Sample aliquots were removed at intervals over a 2-h period and spotted onto PEI-cellulose sheets (Merck). After drying under a light bulb, TLC was performed in 0.2 M ammonium bicarbonate, pH 8.0. Radioactivity in the ATP and the P_i spots was measured by phosphoimager analysis (Applied Biosystems). G-actin ATPase rates were determined by linear curve fitting (Schüler, 2000d).

Wilbanks *et al*., 1994). The stimulation of the actin ATPase activity seen after addition of polymerizing salts is due to the changes in ionic conditions and conformational changes occurring during subsequent incorporation of the actin monomers into filaments.

B. Polymer Formation and ATP Hydrolysis

Filament formation *in vitro* is characterized by a distinct lag phase, the rate-limiting step being the formation of nuclei consisting of three to four actin monomers (Kasai *et al*., 1962). Support for a linkage between ATPase activity and polymer formation has come from experiments in which filament formation was either stimulated by or interfered with different actin-binding proteins, mutations in actin, or actin-modifying reagents (Brenner and Korn, 1980; Tobacman and Korn, 1982; Tellam, 1986; Polzar *et al*., 1989; Geipel *et al*., 1990; Dancker *et al*., 1991; Hayden *et al*., 1993; Kasprzak, 1994; Schüler *et al*., 2000b; Schüler, 2001).

Addition of salts to physiological concentrations is coupled to conformational changes, involving the nucleotide-binding site, making the actin assembly-competent (Higashi and Oosawa, 1965; Rich and Estes, 1976; Rouayrenc and Travers, 1981; Carlier *et al*., 1986; Merkler *et al*., 1987). Kinetic evidence suggest the formation of an intermediate referred to as G*-actin, whose formation by itself does not stimulate the actin ATPase activity. Apparently, the formation of this intermediate depends on the binding of monovalent and divalent cations to a polyanionic surface of the actin molecule (Barany *et al*., 1962; Rouayrenc and Travers, 1981; Pardee and Spudich, 1982). There are also biochemical and physical evidence that the early phase of *de novo* polymerization involves the formation of a special actin dimer, which subsequently seems to be used for filament growth (Steinmetz *et al*., 1997). In view of the results presented in Fig. 4, it would be interesting to use matrix-coupled actin monomers in an attempt to single out the effects of salt on the ATPase activity from the cooperative conformational changes occurring during the incorporation of actin monomers into filaments.

C. The Actin–ATP/ADP·P_i Cap

The actin filament is structurally, as well as functionally, asymmetric, which *in vitro* is reflected in a difference in rate of addition of actin monomers to the two ends. Elongation at the fast polymerizing end [the (+)-end (barbed end)], is 10- to 20-fold faster than at the slow polymerizing end [the (–)-end (pointed end)]. ATP–actin with bound Mg^{2+} has an on-rate that is faster than the on-rate of ADP–actin at both ends, and at the (–)-end ATP–actin dissociates faster than ADP–actin (Bonder and Mooseker, 1983; Lal *et al*., 1984; Pollard, 1986; Selden *et al*., 1986). Thus, during the initial phase of polymerization *in vitro* in the presence of excess ATP, ATP–actin is rapidly and preferentially incorporated into filaments at their (+)-end.

During fast filament elongation, ATP hydrolysis and subsequent P_i release is slower than addition of ATP monomers, resulting in the formation of a detectable ATP/ADP·P_i cap at the (+)-end of the growing filament (Carlier and Pantaloni, 1986; Korn *et al.*, 1987; Pinaev *et al.*, 1995; Melki *et al.*, 1996). It has been argued that hydrolysis occurs preferentially at the boundary between the ATP cap and the ADP·P_i-containing monomers inside the filament (Korn *et al.*, 1987). This would imply that the ADP·P_i–actin monomer has a different structure than the ATP monomer and that the ADP·P_i monomer has a propensity to accelerate ATP hydrolysis on the adjacent ATP monomer. Results reported by others suggest random hydrolysis of ATP within newly formed stretches of the ATP–actin polymer (Ohm and Wegner, 1994; Pieper and Wegner, 1996).

The polymerization reaction does not reach thermodynamic equilibrium. Instead, the different rates of monomer association and dissociation at the two ends eventually result in a steady state in which the net incorporation of ATP–actin at the (+)-end equals the loss of ADP–actin at the (–)-end. As long as there is ATP in the solution, the steady state is characterized by a constant flux of actin monomers through the filaments, a phenomenon referred to as treadmilling (Wegner, 1976; Neuhaus *et al.*, 1983). The rate of treadmilling is determined not only by the combined association and dissociation rate constants at the filament ends but also by the rate of nucleotide exchange on ADP monomers coming off the pointed end. In a solution of purified actin, the nucleotide exchange reaction is the rate-limiting step (Kinosian *et al.*, 1993), and the ATP-actin cap persists at steady state as long as there are ATP-actin monomers available for incorporation.

The atomic structure of the actin filament (F-actin) is not known. Consequently, structural transitions in actin that accelerate ATP hydrolysis also remain to be elucidated. Cryoelectron microscopy has demonstrated that there is a structural difference between ATP/ADP·P_i filaments and ADP-containing filaments, where the latter, that is, the ground state, has the most well-ordered structure (Lepault *et al.*, 1994). A difference between newly formed actin polymers, presumably consisting of ATP/ADP·P_i-actin and filaments consisting of ADP-actin, is further demonstrated by preferential binding of the filament-nucleating Arp2/3 complex to the former *in vitro* (Ichetovkin *et al.*, 2002). ATP hydrolysis and P_i release destabilizes monomer: monomer bonds at filament ends making the ADP polymer dynamic (Rickard and Sheterline, 1986, 1988; and the preceding references).

VI. MECHANISM OF ATP HYDROLYSIS ON ACTIN

A. Active Site Nucleophile

In the actin-related heat shock 70 proteins, ATP hydrolysis likely involves in-line attack on the ATP γ-phosphate by a hydroxyl ion coordinated by K71 (O'Brien *et al.*, 1996; Rajapandi *et al.*, 1998). In actin, the only basic side chains in the vicinity of the γ-phosphate, R177 and H73, have been shown to be nonessential for catalysis by

directed mutagenesis (Schüler *et al.*, 2000b; Nyman *et al.*, 2002). High-affinity Mg^{2+} boosts the ATPase activity of monomeric actin 20- to 30-fold as compared with Ca^{2+} (Geipel *et al.*, 1990; Chen and Rubenstein, 1995; Schüler *et al.*, 1999). Thus, the catalytic activity is regulated via the coordination sphere of the metal cofactor at the base of the cleft. In immediate proximity of the divalent cation, Q137 or H161 may coordinate a hydroxyl ion or water molecule. Structures of nonvertebrate actins suggest that a Q137-bound water molecule may act as a catalytic nucleophile. For a detailed illustration of the catalytic mechanism see Vorobiev *et al.* (2003). A monovalent cation that may coordinate nucleophilic water in Hsc70 (Wilbanks and McKay, 1995) has not been observed in actin.

The location of the hydroxyl of S14 in actin is within hydrogen-bonding distance of the γ-phosphate of ATP, suggesting its involvement in the ATPase reaction. This residue is one of a number of ligands binding to the the γ-phosphate of ATP, thereby stabilizing the actin–ATP complex. In yeast actin, mutation of Ser-14 to Ala (S14A) causes a temperature-sensitive phenotype *in vivo* and temperature-sensitive polymerization defects *in vitro* (Chen and Rubenstein, 1995). It also decreases in the intrinsic ATPase activity of both Ca- and Mg-G-actin at 30 °C and alters the protease susceptibility of sites on subdomain 2. It was proposed that the Ser-14 hydroxyl forms a polar bridge between the ATP γ-phosphate and the amide nitrogen of Gly-74, thus conferring additional stability on the actin small domain.

The mutant S14C in yeast actin does not support growth (Chen and Rubenstein, 1995), but mutant S14C-β-actin can be coexpressed with endogenous yeast actin, and is isolated free of the endogenous protein allowing the investigation of its ATPase activity. The S14C-β-actin retains ATPase activity (Schüler *et al.*, 1999), and Cys-14 in S14C mutant actin reacts covalently with the sulfhydryl of ATPγS (Fig. 5 and Schüler *et al.*, 2000c). This leaves the possibility of a transient phosphoserine formation during the course of ATP hydrolysis. A phosphorylated actin has not been described as an intermediate in the ATPase reaction, but this might be due to instability of such a species. Heat shock 70 proteins are known to undergo autophosphorylation on a threonine residue (T199 in DnaK). However, the function of this reaction and its implications for the mechanism of ATP hydrolysis are still unclear, especially since they seem to vary between members of the protein class (McCarty and Walker, 1991; Gaut and Hendershot, 1993; O'Brian and McKay, 1993; Barthel *et al.*, 2001). Therefore, it is possible that actin with phosphoserine at position 14 is an intermediate in a switch mechanism partitioning the release of free energy after ATP hydrolysis.

B. Catalytic Base(s)

In many phosphoryl transferases, the active site nucleophile is activated by a nearby side chain. Mutational analyses of Hsp70 proteins have shown that not only the glutamate or aspartate in the position corresponding to actin Q137 but also other nearby carboxylic side chains are important for full catalytic activity (Gaut and Hendershot, 1993; McCarty and Walker, 1994; Wilbanks *et al.*, 1994; Kamath-Loeb

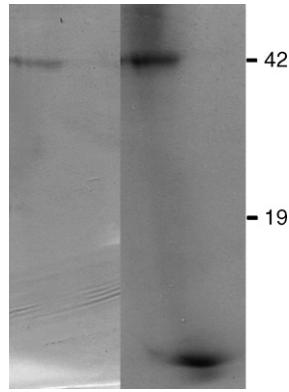

Figure 5. Covalent binding of ATPγS to S14C-actin. Monomeric Mg-actin (10 μM) carrying the S14C replacement was incubated with 0.1 mM of ATPγ-^{35}S for 1 h. Excess nucleotide was removed by gel filtration before the protein was denatured by addition of 2% SDS and subjected to SDS-PAGE under nonreducing conditions. An autoradiograph (right lane) of the Coomassie-stained gel (left lane) showed that approximately one-third of the total radioactivity resided in the protein band, illustrating that a transfer of the ^{35}S from the nucleotide to the protein had occurred, most likely to cysteine at position 14.

et al., 1995). Actin Q137 is unlikely to be deprotonated under physiological conditions and is therefore a poor base. Given that the ATPase reaction might proceed without conserved symmetry, residues D11 and D154 could be catalytic bases. Semiconservative replacements of D11 in yeast actin lead to mild defects, whereas charge reversions as well as the double replacement D154A, D157A are lethal (Cook *et al.*, 1992, 1993; Wertman *et al.*, 1992). The ATPase activities of these mutant proteins, however, have not been tested.

The replacement V159N in yeast actin causes an increased release of inorganic phosphate and a high rate of filament turnover, while ATP hydrolysis itself seems unaffected (Belmont *et al.*, 1999). These results were interpreted as an uncoupling of P_i release from a conformational change, which destabilizes the actin filament. Thus, V159 is necessary for harnessing the free energy change of P_i release for a discrete step. In actin V159 is conserved except for a few sequences that have isoleucine in this position, while in the 70-kDa heat shock proteins a threonine is highly conserved in the corresponding position. This small hydrophobic barrier shielding the γ-phosphate from solvent seems to have evolved as a special feature for actin.

VII. ACTIN METHYLHISTIDINE 73, ATPASE, PHOSPHATE RELEASE, AND POLYMERIZATION

Saccharomyces cerevisiae, which is used for the expression of wild-type and mutant β-actin, does not methylate histidine 73 (Yao *et al.*, 1999). This was utilized in setting up a series of experiments to elucidate the role of the histidine as well as its

methylation in ATP hydrolysis and P_i release and polymerization of the actin. For this, wild-type β-actin (with MeHis73) isolated from calf thymus, β-actin expressed in yeast (nonmethylated), and mutant H73A β-actin also expressed in yeast were used (Nyman *et al.*, 2002). As shown in Fig. 6A, bovine β-actin hydrolyzed ATP only slightly ahead of polymerization and phosphate release, whereas β-actin expressed in yeast (nonmethylated) hydrolyzed ATP and released P_i well ahead of polymer formation (Fig. 6B). These results were at odds with earlier work reporting a sequence of events in which ATP hydrolysis and polymer formation went hand in hand, whereas P_i release was significantly delayed (Carlier *et al.*, 1986; Melki *et al.*, 1996). To clarify this, experiments were performed with rabbit skeletal muscle actin (α-actin). As shown in Fig. 6C, α-actin (methylated) released P_i only after polymers had formed, corroborating earlier results.

Comparison of β-actin expressed in yeast (nonmethylated) with the mutant H73A-β-actin also expressed in yeast showed that in both cases hydrolysis of ATP and P_i release preceded polymer formation, suggesting an uncoupling of the hydrolysis and product release from filament formation and that most likely in these cases polymers form from actin monomers with ADP on them. This is reasonable, since actin with bound ADP (Higashi and Oosawa, 1965; Kasai *et al.*, 1962; Pollard, 1984) and actin with nonhydrolyzable nucleotide analogues (Cooke and Murdoch, 1973) can polymerize. Thus, neither ATP hydrolysis nor bound ATP is needed for polymerization to occur, although there may be significant differences in the quality of the filament formed from the different actin states. The H73A mutant β-actin did not form filaments in the absence of Mg^{2+} ions and ATP hydrolysis was very slow (Nyman *et al.*, 2002). The nonmethylated β-actin did form filaments in the absence of Mg^{2+}, albeit at a slow speed, further emphasizing the importance of a histidine in position 73 in keeping the actin in a polymerizable state. The difference in the kinetics of polymer formation suggests that proper polymers form only with actin having a MeH73 in it. It should also be noted that there is a clear isoform difference in that α-actin holds on to the P_i much longer than native β-actin does, something which may be related to a difference in force generation in the highly organized myofibrillar actomyosin system as compared with the less stable MFS in nonmuscle cells, where instead rapid actin reorganization is crucial for the function (Nyman *et al.*, 2002).

As shown in Fig. 1, MeH73 and D184 have moved apart in the open state of the β-actin, allowing D184 to form a salt bridge with R183 rather than H73, and R177 of β-actin has moved from hydrogen bonding with the backbone atoms of MeH73 to a salt bridge interaction with the D179. In the presence of Mg^{2+} ions, the actin ATPase activity is greatly stimulated. Under these conditions, the region near Y69 (R62–K68) is protected from proteolysis (Strzelecka-Golaszewska *et al.*, 1993), suggesting that the interdomain cleft is closed. Thus, it is possible that the binding of Mg^{2+} ions stabilizes the tight state of actin allowing nucleotide hydrolysis to take place. Following ATP hydrolysis, breakage of the interdomain bridges might allow the opening of the cleft, facilitating release of the γ-phosphate directly into the solvent. Such a mechanism is supported by the fact that actin can attain a state in which the interdomain cleft is opened up (Chik *et al.*, 1996). It suggests that changes in the conformation of

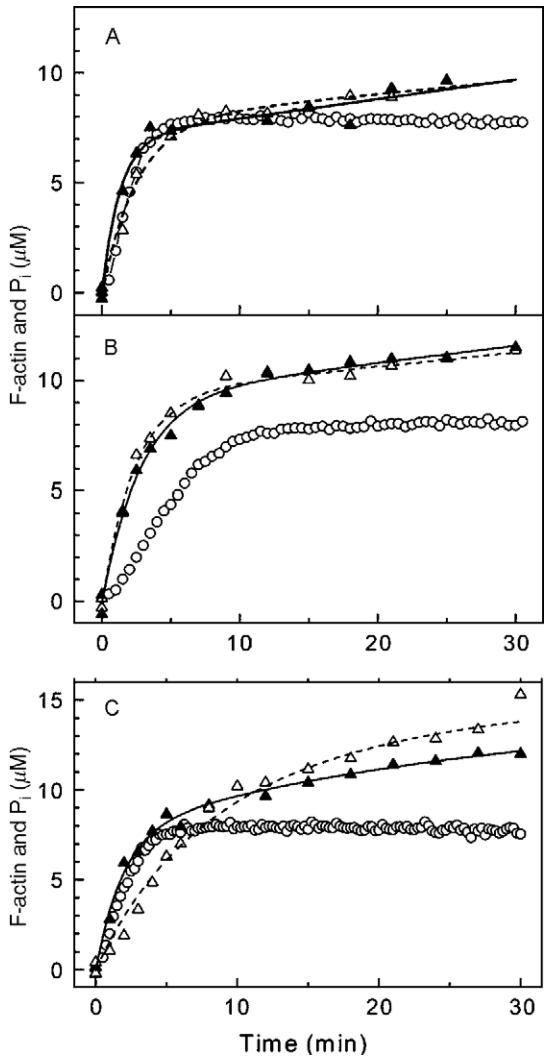

Figure 6. Comparison of native β-actin (MeHis73) with yeast-expressed β-actin (H73) and native α-actin with respect to ATP hydrolysis, phosphate release, and polymerization. Polymerization of Mg-actin (8 μM) was induced by 0.1 M KCl and 1 mM $MgCl_2$ and monitored using the pyrenyl assay (open circles). Samples were withdrawn at the time points indicated and analyzed for ATP hydrolysis (closed triangles) and P_i release (open triangles) as described earlier (Nyman *et al.*, 2002). (A) β-Actin from bovine thymus (methylated), (B) yeast-expressed β-actin (unmethylated), and (C) α-actin (methylated). (C) The measured amount of P_i released exceeds the total amount of ATP hydrolyzed. This anomaly depends on the experimental design and does not invalidate the conclusion. In fact, the delay in the P_i release from α-actin would be even more pronounced if the anomaly was corrected. Figure published in (2002) *J. Mol. Biol.* **317,** 577–589.

actin opens the interdomain cleft to allow the inorganic phosphate to escape directly into solution rather than through a narrow "backdoor" channel (Wriggers and Schulten, 1997).

VIII. IMPORTANCE OF THE STATUS OF THE ACTIN-BOUND NUCLEOTIDE

Only little information is available about the conformation of actin monomers in filaments formed under different conditions, but it is generally believed that hydrolysis of ATP on actin *in vivo* has a regulatory function in determining the structure and the dynamic turnover of actin monomers and their interactions with actin-binding proteins. In fact, actin depolymerizing factors (ADF/cofilins) (Carlier *et al*., 1997; Blanchoin and Pollard, 1998), twinfilin (Palmgren *et al*., 2001), adenylate cyclase-associated protein (CAP; Mattila *et al*., 2004), and gelsolin (Laham *et al*., 1993) bind preferentially to ADP–actin, while DNase I (Schüler *et al*., 2000c), profilin (Vinson *et al*., 1998), thymosin β4 (Carlier *et al*., 1993), Arp2/3 (Ichetovkin *et al*., 2000), and Wiskott–Aldrich syndrome protein (WASP) homology domain 2 (Chereau *et al*., 2005) bind preferentially to ATP–actin. Binding of ADF–cofilin to actin filaments is strongly enhanced by P_i release (Blanchoin and Pollard, 1999). Thus, ATP hydrolysis is an integral part in the regulation of the function of actin and actin filaments *in vivo*.

In cells, the ultimate precursor in the formation of actin filaments is profilin:actin, and polymerization takes place either onto preexisting free filament ends or at specific sites formed by polymerization-promoting protein complexes (Hajkova *et al*., 2000; Grenklo *et al*., 2003; Higashida *et al*., 2004; Romero *et al*., 2004). Profilin effectively accelerates the exchange of the nucleotide on actin by opening up the nucleotide-binding cleft (Chik *et al*., 1996). Also mutant profilins, which bind actin only weakly, stimulate nucleotide exchange (Korenbaum *et al*., 1998). Profilin binds more strongly to actin–ADP than cofilin. Therefore, profilin efficiently forms profilin–ATP–actin from the cofilin–ADP–actin, which comes off the (–)-end of depolymerizing filaments. Profilin also inhibits the ATPase activity of the actin monomer, and thus ensures the delivery of ATP–actin for incorporation into growing filaments by actin-polymerizing machineries (Dickinson *et al*., 2002). Thus, treadmilling occurs *in vivo* and is at the heart of myosin-independent, actin-based translocations, and the loss and addition of actin monomers is strictly regulated by auxiliary proteins.

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