Functional Significance of the Binding of One Myosin Head to Two Actin Monomers†

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ABSTRACT: The functional significance of the interaction of one myosin head (S1) with two actin monomers was investigated by comparing the properties of the cross-linked monomeric and filamentous actin−S1 complexes. S1 was cross-linked to monomeric actin (G-actin) either in the absence or in the presence of DNase I by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. The binary G-actin−S1 and ternary DNase I−G-actin−S1 complexes were then purified by a combination of ion exchange and gel filtration columns. Both the binary and the ternary complexes were characterized by negligible, though different, Mg^{2+}-ATPase activities of 0.018 and 0.006 s^{-1}, respectively. Using fluorescence, light-scattering, and ultracentrifugation experiments, we found that only the binary G-actin−S1 complex could be polymerized in the presence of 2 mM $MgCl_2$. Electron microscopic analysis of the cross-linked filamentous complex showed fully decorated filaments with the arrowhead pattern characteristic of the non-covalent complex in the rigor state. Such a 100% cross-linked F-actin−S1 complex exhibited a Mg^{2+}-ATPase activity of 6.2 ± 0.8 s^{-1}, slightly lower than the maximum velocity of the non-cross-linked complex of 8.6 ± 0.8 s^{-1}, but comparable to the 6.9 ± 0.6 s^{-1} obtained for a partially (35%) cross-linked complex. These results implied that the activation of S1 ATPase by actin requires the interaction of S1 with a second actin monomer within the thin filament. They also suggested that the full activation of the filamentous complex is not dependent on the degree of saturation of the thin filament by myosin.

Muscle contraction and cell motility processes use ATP[1] hydrolysis as energy supplier. In the case of the actin−myosin system, the myosin ATPase is dramatically activated (up to 500-fold) when interacting with filamentous actin (F-actin) [see, for exemple, Eisenberg et al. (1968)]. On the other hand, monomeric actin (G-actin) does not affect significantly myosin or myosin subfragment 1 (S1) ATPase (Offer et al., 1972; Estes & Gershman, 1978; Lheureux & Chaussepied, 1995).

In the accompanying paper (Lheureux & Chaussepied, 1995) we demonstrated that this lack of ATPase activation is due to a very low affinity of G-actin for the S1−nucleotide intermediates (Lheureux & Chaussepied, 1995). In fact, the contacts between G-actin and S1 appear to resemble the ionic contacts involved in the collision F-actin−S1 complex and hence they would not be sufficient to create the weak or the strong binding states taking place between F-actin and S1 during the ATP hydrolysis cycle (Lheureux & Chaussepied, 1995).

Like the collision complex, the G-actin−S1 interface is mostly electrostatic in nature, encompassing the myosin loop 626−646 (containing the positively charged segment 636−642) and the negative patch of actin residues Asp1-Glu4, Asp24-Asp25, and Glu99-Glu100 (Lheureux et al., 1993; Lheureux & Chaussepied, 1995). On the other hand, the hydrophobic contacts between actin residues Ala144, Ile341, Ile345, Leu349, Phe352, and S1 residues Pro529, Met530, Ile535, Met541, Phe542, and Pro543 or between actin segment 332−334 and S1 segment 403−415 proposed by Rayment et al. (1993) and Shröder et al. (1993) seem significantly impaired in the monomeric complex (Lheureux et al., 1993). It was also found that the G-actin−S1 interface does not implicate the contacts between the lower 50-kDa domain of S1 and actin subdomain 2 (Bonafé & Chaussepied, 1994) or actin segment 89−93 (or N-terminal segment 1−28 for unsaturated filaments; Bonafé & Chaussepied, 1995) of the adjacent monomer in the thin filament.

It is easily understandable that the contacts between S1 and a second actin monomer take place in the filamentous complex but not in the monomeric 1:1 G-actin−S1 complex. However, it is surprising that the hydrophobic sites are not accessible to S1 though they are located on the same monomer as the electrostatic sites. Due to the importance of these hydrophobic contacts in ATPase activation and force production (Rayment et al., 1993; Holmes, 1995; Geeves & Conibear, 1995), it is essential to understand how they are created in the F-actin−S1 complex.

Two hypotheses could account for the presence of hydrophobic contacts in the filamentous but not in the monomeric complexes. These two hypotheses are characterized by the relative role of S1 binding to the second actin monomer.

In the first hypothesis, S1 binding to the hydrophobic sites would be the result of structural changes at the junction

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[1] Abbreviations: S1, myosin subfragment 1; S1(A2), S1 isoenzyme with alkaline light chain 2; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide; NHS, N-hydroxysuccinimide; ADP and ATP, adenosine di- and triphosphate; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PAGE, polyacrylamide gel electrophoresis.

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between actin subdomains 1 and 3 during actin polymerization. In this case the interaction between S1 and the second actin monomer in the filament would be of minor importance and could have a role, if any, in stabilizing the final rigor complex. Very few arguments sustain this possibility since all of the refinements used so far to fit the actin monomer within the filament lattice have revealed little or no change in the structure of this actin region after polymerization (Holmes et al., 1990; Lorenz & Holmes, 1993; Orlova & Egelman, 1993; Tirion & ben-Avraham, 1993; Mendelson & Morris, 1994; Bremer et al., 1994; Tirion et al., 1995). Moreover, normal mode analysis of monomeric as well as filamentous actin revealed that this region is characterized by the lack of internal motion which makes it very unlikely to be altered by S1 binding (Tirion & ben-Avraham, 1993; Tirion et al., 1995).

In the second hypothesis, S1 has to interact with the second monomer in order to bring close to each other the two hydrophobic counterparts. In this case, S1 binding to the second monomer would be crucial for the formation of the weak and possibly of the strong binding states. The interaction of S1 with the second monomer has been experimentally established with actin subdomain 2 regardless of the actin:S1 ratio (Bonafé et al., 1994) and with actin segment 1−28 only at actin:S1 ratios higher than 1 (Andreev & Borejdo, 1992; Bonafé & Chaussepied, 1995). However, there is no evidence implicating these interactions in the ATPase and force generation processes.

In the present study we examined the role of S1 binding to a second actin monomer in the filamentous complex by comparing the properties of the cross-linked actin−S1 complexes in their monomeric and filamentous forms. For this purpose, we took advantage of the fact that a soluble and monomeric form of the actin−S1 complex could be isolated at low ionic strength with the use of S1(A2) isozyme, i.e., S1 carrying the alkali light chain 2 (Chaussepied & Kasprzak, 1989; Chen & Reisler, 1991; Lheureux et al., 1993, 1995; Kasprzak, 1993, 1995). In addition, we used the cross-linking approach to overcome the low affinity of G-actin for the S1−nucleotide intermediates. Indeed, the cross-linking reactions were found to generate filamentous complexes in which S1 spends essentially all of its time bound to actin even in the presence of ATP. Consequently, the ATPase rate of the covalent filamentous complex approximates the maximum rate obtained at infinite actin concentration (Mornet et al., 1981; Greene, 1984; Biosca et al., 1985; King & Greene, 1985; Bertrand et al., 1988; Bonafé et al., 1993). The covalent monomeric complex was produced by EDC-induced cross-linking and purified by ion exchange and gel filtration columns, and its ATPase activity was measured before and after polymerization. The results strengthened the idea that S1 binding to a second actin monomer is crucial for ATPase activation. During this work, we also compared the ATPase activities of filamentous actin saturated and non-saturated by cross-linked S1. It was thus possible to evaluate the functional significance of the degree of saturation of the thin filament by S1.

MATERIALS AND METHODS

Reagents. α-Chymotrypsin was from Worthington Biochemicals. ATP, soybean trypsin inhibitor, and DNase I were obtained from Boehringer Mannheim. Hydroxyapatite was from Serva. EDC and NHS were obtained from Sigma. Sephacryl S-200 and Sephadex G-150 were obtained from Pharmacia. *N*-(1-Pyrenyl)iodoacetamide was from Molecular Probes. All other chemicals were of the highest analytical grade.

Preparation of Proteins. Rabbit skeletal myosin was prepared as described by Offer et al. (1973). S1 was obtained by chymotryptic digestion of myosin filaments (Weeds & Taylor, 1975). S1(A1) and S1(A2) were separated by ion-exchange chromatography on SP-Trysacryl-M (Lheureux et al., 1993). Rabbit skeletal F-actin was prepared as described by Eisenberg and Kielley (1974). G-actin was obtained by depolymerization of F-actin and purified over Sephacryl S-200 as described by Lheureux and Chaussepied (1995). F-actin was obtained by polymerization of gel-filtered G-actin in the presence of 100 mM KCl and 2.5 mM $MgCl_2$ at 30 °C for 2 h. F-actin was kept on ice for less than a week in buffer F (5 mM HEPES, 0.1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 100 mM KCl, and 0.1 mM NaN_3, pH 8.0).

Pyrenyl-labeled G-actin (pyr-G-actin) was prepared according to Cooper et al. (1983) and further modified by Lheureux and Chaussepied (1995).

Commercial DNase I from bovine pancreas was purified as described by Price et al. (1969) and Lheureux et al. (1993).

Protein concentrations were determined spectrophotometrically as described (Lheureux & Chaussepied, 1995).

Cross-Linking Reactions. The covalent G-actin−S1(A2) and DNase I−G-actin−S1(A2) complexes were generated by the two-step cross-linking reaction as proposed by Grabarek and Gergely (1990) and Sehgal and Vijay (1994). First, G-actin (15−20 μM) eluted from an S-200 column was supplemented by 10 mM MOPS, pH 7.0. The activation step was initiated at 4 °C by 2.5 mM EDC and NHS. After a 5-min reaction, the excess of cross-linker was quenched by the addition of 5 mM β-mercaptoethanol. Second, S1(A2) (1.2-fold excess over actin) with or without DNase I (1.5-fold excess over actin) was added to activated G-actin, and the reaction mixture was centrifugated at 180000*g* for 1 h at 4 °C to remove actin oligomers formed during the condensation step. The supernatant, containing both covalent and non-covalent G-actin−S1(A2) complexes, was filtered through 0.22-μm pore size Millipore membrane, and ATP was added to a final concentration of 2.5 mM in order to dissociate non-cross-linked S1.

In the case of the binary G-actin−S1 complex, the excess of S1(A2) was eliminated by an SP-Trysacryl M (5 × 10 cm) column equilibrated with buffer G (supplemented by 2 mM ATP). The S1-free flow-through fraction was gently concentrated at 4 °C on an Amicon Cell (PM30 membrane), and the final 10−15-mL solution was loaded on a Sephadex G-150 column (2.7 × 120 cm) equilibrated with buffer G. The content of the different column fractions was assessed by gel electrophoresis. The purified complex was kept on ice and used within 3 days.

In the case of the ternary DNase I−G-actin−S1(A2) complex, the ATP-containing solution of cross-linked proteins was concentrated in an Amicon Cell (PM30 membrane). Cross-linked and non-cross-linked proteins were then separated by loading at most 1 mL of the protein solution on an HPLC gel filtration setup composed of a TLK 3000 and a TLK 4000 column mounted in series on a Beckman Model 110B HPLC system and equilibrated with buffer A (10 mM MOPS, 100 mM KCl, 0.2 mM ATP, 0.2 mM $CaCl_2$, and

0.1 mM NaN_3, pH 7.0) at a flow rate of 1 mL/min. The purity of the DNase I−G-actin−S1(A2) complex was then tested by gel electrophoresis.

The covalent F-actin−S1 complex was generated as described for the binary complex except that polymerization of the complex was induced by 2 mM $MgCl_2$ and 120 mM KCl for 1 h at 25 °C just after the centrifugation step. Non-cross-linked S1 was then dissociated with 1 vol of 100 mM Tris, 500 mM NaCl, 1.6 M KCl, 20 mM $Na_4P_2O_7$, 10 mM $MgCl_2$, and 10 mM ATP, pH 8.0, followed by a centrifugation at 380 000*g* for 20 min at 4 °C. The yield of the cross-linking reaction (which varied from 30 to 40%) and the amount of residual non-cross-linked S1 (about 5% of the total S1) were then determined on triplicate samples by quantitative gel electrophoresis as described (Herrman et al., 1993).

Concentration of the cross-linked complexes was determined spectrophotometrically with extinction coefficients of $A^{1\%}_{280nm}$ = 9.2 and 10.3 cm^{-1} for the binary G-actin−S1 (MW = 147) and the ternary DNase I−G-actin−S1 (MW = 178) complexes.

EDC-treated actin (EDC-actin) was obtained as described for the binary complex except that S1(A2) was not added to the reaction mixture. It is important to note that, due to the two-step cross-linking procedure, actin modification was similar among the various cross-linked adducts generated in this study.

Gel Electrophoresis. The purity of the proteins was verified by 3−18% polyacrylamide gradient gel electrophoresis in the presence of 0.1% SDS as described by Laemmli (1970).

Spectral Measurements. Fluorescence and light-scattering measurements were carried out as described (Lheureux et al., 1993).

ATPase Measurements. The Mg^{2+}-ATPase activities of the actin−S1 complexes were determined at 20 °C in 10 mM Hepes, 0.1 mM $CaCl_2$, and 0.12 mM EGTA, pH 7.6, in the presence of various concentrations of Mg^{2+}-ATP, by two different methods depending on the total amount of protein available. With protein samples in large quantities, we used the colorimetric method as described (Mornet et al., 1981). With protein samples in lower quantities, we followed the ATP-induced change in the fluorescence signal of the pyrenyl moiety attached to actin. In this latter case, the activities were calculated as in Lheureux and Chaussepied (1995).

Electron Microscopy. Samples containing less than 0.1 mg/mL of filamentous actin were mounted on Formar/carbon-coated grids pretreated with cytochrome *c* and negatively stained by 2% uranyl acetate (Craig & Negerman, 1977). Grids were examined using a JEM 200 CX microscope operating at an accelerating voltage of 80 kV.

RESULTS

Purification of the Cross-Linked G-Actin−S1 Complexes. Previous studies showed that depolymerization of the cross-linked F-actin−S1 complexes could be achieved only after a partial denaturation of actin by EDTA (Greene, 1984; Rouayrenc et al., 1985; Bertrand et al., 1988). Therefore, the only way to isolate the cross-linked G-actin−S1 complexes in a native form was to initiate the reaction on the monomeric complexes. Chaussepied and Kasprzak (1989) succeeded in this task only by cross-linking G-actin to S1 at

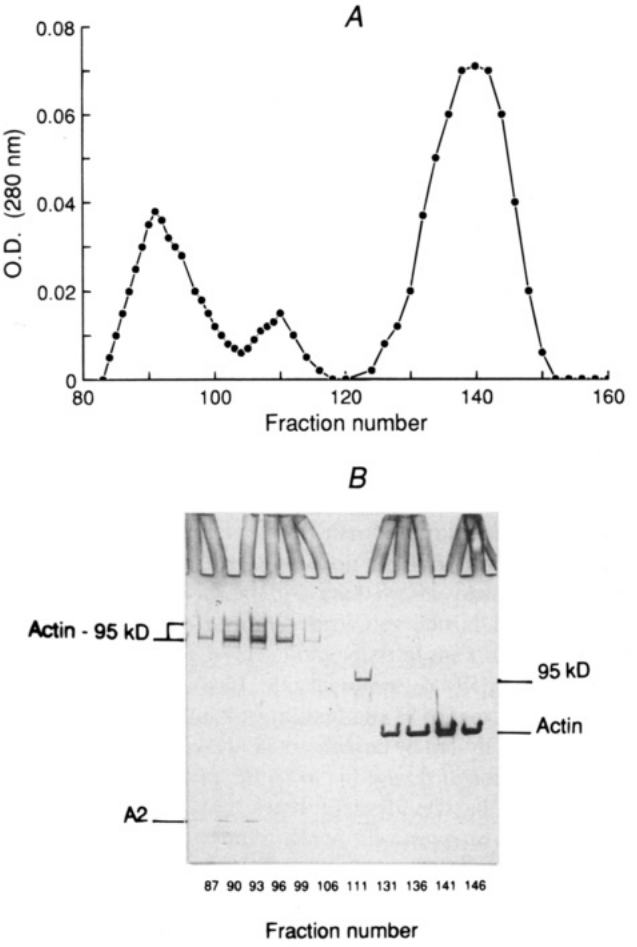

FIGURE 1: Purification of the cross-linked G-actin−S1(A2) complex. (A) Elution profile from a Sephadex G-150 column. (B) SDS−PAGE analysis of column fractions 87−146.

4°C in a weak ionic strength buffer. In fact, these conditions were necessary to prevent polymerization of G-actin. While keeping the same temperature and ionic strength conditions, we used here a slightly modified method which markedly improved the yield as well as the purity of the covalent G-acto-S1 complexes.

First of all we employed a two-step reaction in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) (Grabarek & Gergely, 1990). NHS was added to increase the yield of the EDC reaction and the use of the two-step reaction allowed avoidance of the uncontrolled modification of S1. Second, the purification step was improved by adding an ion-exchange chromatography which removed most of the non-cross-linked S1 from the reaction mixture. As a consequence, the elution pattern of the subsequent G-150 chomatography presented in Figure 1 was at variance with that previously published (Chaussepied & Kasprzak, 1989) since the three main sets of fractions were also found but with different relative intensities (Figure 1A). Fractions 80−105 corresponded to the acto-S1(A2) cross-linked complex, which migrated on gel electrophoresis as a doublet with apparent masses of 165−200 kDa and was composed of 1 mol of G-actin covalently linked to 1 mol of S1 heavy chain (Combeau et al., 1992); fractions 106−118, which contained the remaining non-cross-linked S1(A2); and fractions 120−150 almost exclusively composed of actin (Figure 1B). The intensities of the S1-containing fractions were significantly

reduced as compared with the elution profile of Chaussepied and Kasprzak (1989). This resulted in the purification of covalent complexes free of contaminating S1(A2). Our final preparation was also free of non-cross-linked actin in contrast to the data reported by Combeau et al. (1992). These last authors interpreted their results as evidence for the interaction of two molecules of actin, one of which was cross-linked with one molecule of S1. The absence, in our preparation, of free actin confirmed the idea that we cross-linked and purified a stoichiometric covalent G-actin−S1(A2) complex identical to the stoichiometric non-covalent complex that we and others previously characterized (Chaussepied & Kasprzak, 1989; Chen & Reisler, 1991; Lheureux et al., 1993; Kasprzak, 1993, 1994).

Isolation of the ternary cross-linked DNase I−G-actin−S1(A2) complex was also of great interest since DNase I, which blocks the polymerization of the reversible complex (Lheureux et al., 1993), could allow the study of the covalent complex under polymerizing conditions. The cross-linked ternary complex was generated by EDC reaction as described for the covalent binary complex except that DNase I was added simultaneously to S1(A2) prior to the condensation step. Adding DNase I early in the reaction and not at the end of the purification step had the advantage of increasing the yield of the reaction since only trace amounts of actin oligomers were then sedimented during the ultracentrifugation step. It also had the advantage of allowing a more rapid purification using a one-step HPLC gel-exclusion column in the presence of 100 mM KCl and 0.2 mM ATP. Analysis of the elution profile of the HPLC chromatography revealed two main parts (Figure 2). The first set of fractions contained DNase I and the cross-linked acto-S1(A2) adducts similar to those obtained in the absence of DNase I (compare Figures 1 and 2). As in the case of the binary complex, no trace of free actin or S1 heavy chain was contaminating this ternary DNase I−G-actin-S1(A2) complex. The second set of fractions was composed of the non-cross-linked proteins, DNase I, G-actin, and S1(A2). It was previously demonstrated that the presence of 0.1 M KCl and 0.2 mM ATP was sufficient to dissociate S1 but not DNase I from G-actin (Lheureux et al., 1993; Lheureux & Chaussepied, 1995). Therefore, it is most probable that the two protein entities really present in these fractions were S1(A2) (mol mass = 105 kDa) and the DNase I−G-actin complex (mol mass = 73 kDa), which could not be separated on this type of HPLC column.

Polymerization Properties. Non-cross-linked G-actin−S1-(A2) complex was found to remain in the monomeric form, in low ionic strength buffer and at 20 °C, as long as its concentration was maintained lower than 8 μM (Lheureux et al., 1993).

In comparison, the binary and ternary covalent complexes could be kept in a monomeric state (under similar low-salt conditions) at protein concentrations of 3 μM as determined by ultracentrifugation experiments (data not shown). Unfortunately, higher protein concentrations could not be tested due to the low amount of pure covalent complex obtained by this method. On the other hand, the cross-linked G-actin−S1(A2) complex was able to polymerize at concentrations lower than 1 μM in the presence of salts. Figure 3A describes a typical time course of polymerization of the binary complex induced by 2 mM $MgCl_2$. Light scattering and fluorescene intensity of pyrenyl moiety attached to actin

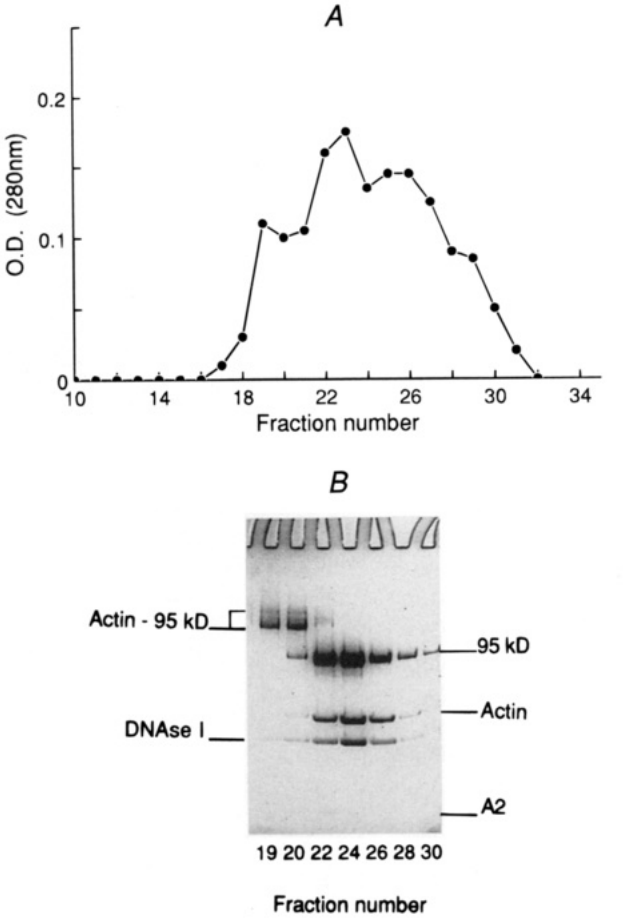

FIGURE 2: Purification of the covalent DNase I−G-actin−S1(A2) complex. (A) Elution profile from HPLC gel-filtration columns. (B) SDS−PAGE of column fractions 19−30.

both slowly increased as the polymerization proceeded. The 1.5−2-fold fluorescence increase observed at the end of the polymerization was lower than the 2.5-fold increase obtained after polymerization of the non-cross-linked G-actin−S1-(A2) complex (Figure 3B; Lheureux et al., 1993). However, this difference seemed to be due to EDC treatment since the polymerized form of EDC-treated actin displayed a similarly low fluorescence enhancement (compare the final plateau in Figure 3B), while the amount of polymerized actin was identical (higher than 90%) in both cases as judged by the amount of monomeric actin in the supernatant of ultracentrifugation experiments (data not shown).

As control experiments, EDC treatment of actin did not diminish S1 binding to G-actin since addition of S1(A2) to pyr-G-actin induced the same fluorescence enhancement as with unmodified actin; neither did it affect the rate of actin polymerization achieved after 20 min in both cases (Figure 3B).

The rate of polymerization of the cross-linked complex was slower than that of the non-cross-linked complex (measured under identical experimental conditions, Figure 3). In the former case, 120 min was not sufficient to attain the plateau, while in the latter case, polymerization was completed after a 20-min reaction. Note that at least 90% of the actin present in the solution was polymerized in both samples. A slower rate of polymerization for the cross-linked complex was surprising since the permanent binding of S1-(A2) to G-actin should have amplified rather than reduced the accelerating effect of S1 on actin polymerization (Chen

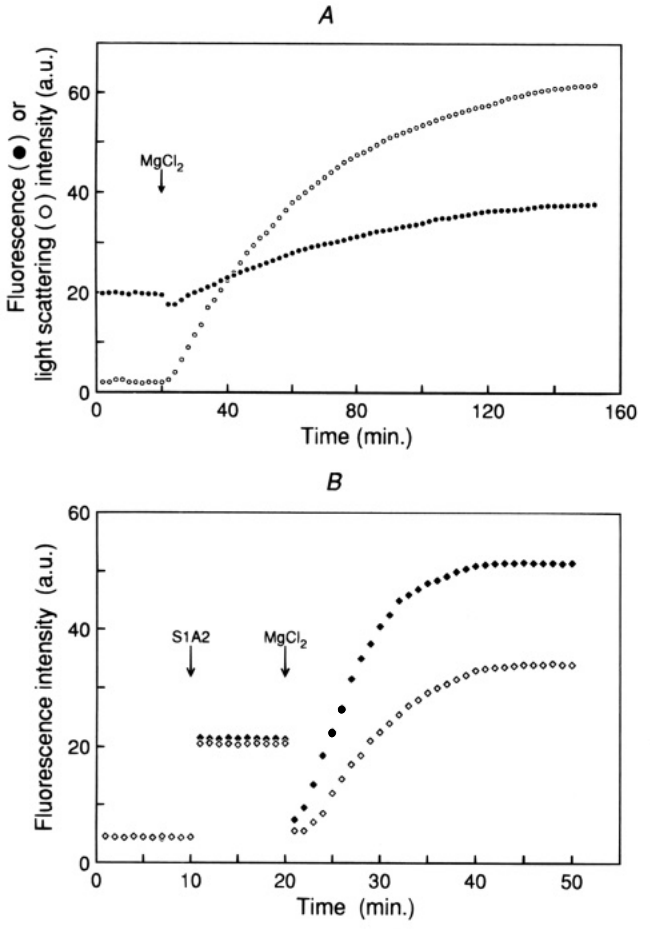

FIGURE 3: Time course of polymerization of the cross-linked (A) and the reversible (B) G-actin−S1(A2) complexes. (A) Polymerization of 0.8 μM cross-linked G-actin−S1(A2) complex monitored by following the change of light scattering (○) and pyrenyl fluorescence (●) as described under Materials and Methods. At the time indicated, 2 mM $MgCl_2$ was added. (B) Polymerization of 0.8 μM G-actin (◆) or EDC-G-actin (◇) monitored by the change of the pyrenyl fluorescence. At the times indicated, 8 μM S1(A2) and 2 mM $MgCl_2$ were successively added. In all cases, polymerization was performed at 20°C in buffer G containing 0.5 μM ATP.

& Reisler, 1991). One possible explanation could be that the cross-linking of S1 to actin either sterically impeded the formation of the actin−actin contacts or slowed down the salt-induced conformational changes on actin. Both of these possibilities were supported by the fact that the salt dissociating effect of the actin−S1 interaction (as reported by the drop of fluorescence upon addition of $MgCl_2$) was larger for the non-cross-linked (Figure 3B) than for the cross-linked (Figure 3A) complex.

Finally, electron microscopic analysis of the polymerized cross-linked actin−S1(A2) complex showed fully decorated filaments with the arrowhead pattern characteristic of the non-covalent complex (Figure 4; Huxley, 1969). It is important to note the absence of actin bundles in all of the samples analyzed. The lack of actin bundles with 100% cross-linked F-actin−S1 complex was in fact in total agreement with the fact that only partly decorated filaments were found to generate such nonphysiological structures (Ando & Scales, 1985; Bonafé & Chaussepied, 1995).

In contrast to the covalent G-actin−S1(A2) complex, the ternary cross-linked DNase I−G-actin−S1(A2) complex did not polymerize as suggested by light scattering and fluorescence measurements or by ultracentrifugation and electron microscopy experiments (data not shown). This result confirmed the inhibitory effect of DNase I as reported on the polymerization of the non-covalent complex (Lheureux et al., 1993).

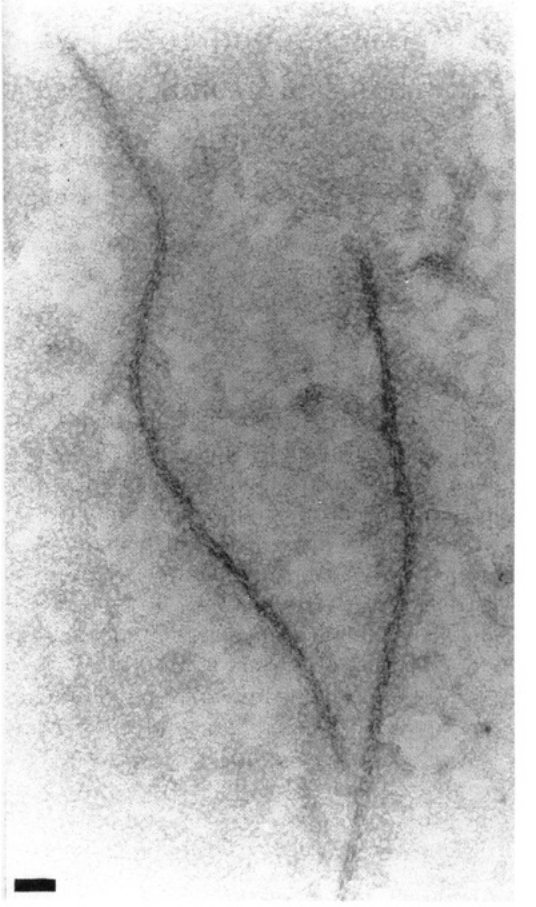

FIGURE 4: Electron micrograph of the cross-linked acto-S1(A2) complex polymerized by 2 mM $MgCl_2$. Scale bar represents 0.1 mm.

ATPase Activities. The Mg^{2+}-ATPase activities of the cross-linked actin−S1 complexes were measured by monitoring the effect of Mg^{2+}-ATP on the fluorescence of the pyrenyl-labeled complexes. As shown in Figure 5A, addition of Mg^{2+}-ATP to a solution containing the monomeric cross-linked G-actin−S1(A2) complex induced an instantaneous 3-fold decrease of the fluorescence intensity similar to the effect observed when the non-cross-linked complex was dissociated by the nucleotides (Valentin-Ranc et al., 1991; Lheureux et al., 1995) or by salts (Lheureux et al., 1993). This first drop was followed by a steady state corresponding to the hydrolysis of Mg^{2+}-ATP by S1. As the Mg^{2+}-ATP hydrolysis was completed, the signal slowly increased to a level lower than the initial fluorescence intensity. This lower final value was interpreted as a partial dissociation of the complex induced by the products of the ATPase reaction, ADP and P_i, as reported for the non-cross-linked complex (Lheureux & Chaussepied, 1995). Similar results were obtained with the cross-linked DNase I−G-actin−S1(A2) complex (data not shown).

Similarly, the fluorescence of the polymerized covalent actin−S1(A2) complex was sensitive to Mg^{2+}-ATP, though in a totally opposite manner (Figure 5B). Indeed, addition of Mg^{2+}-ATP to a solution of cross-linked F-actin−S1(A2)

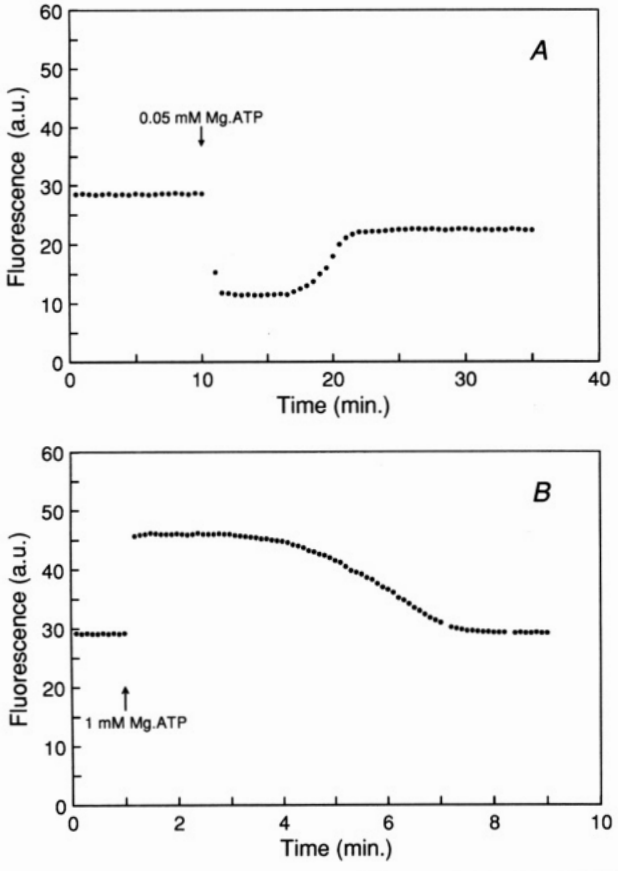

FIGURE 5: Mg^{2+}-ATPase activities of the covalent actin−S1(A2) complexes in the monomeric (A) and filamentous (B) forms. Mg^{2+}-ATPase was monitored by following the change in pyrenyl fluorescence attached to actin as described under Materials and Methods. At the arrows, 50 μM and 1 mM Mg^{2+}-ATP were added to 0.8 μM G-actin-S1(A2) and F-actin-S1(A2), respectively.

complex resulted in a 1.5-fold increase of the fluorescence intensity which, in fact, also corresponded to the Mg^{2+}-ATP dissociation effect observed with the non-cross-linked filamentous complex (Miller et al., 1988). As with the monomeric complex, this rapid increase was followed by a steady state, characterized by a stable fluorescence signal which ended as the ATP hydrolysis was completed by the progressive return to the initial fluorescence value. Note that an ADP + P_i concentration of 1 mM did not apparently dissociate the F-actin−S1(A2) complex, while the same hydrolysis products at 50 μM significantly dissociated the G-actin−S1(A2) complex. This different sensitivity to nucleotide binding confirmed the results obtained with the non-cross-linked complex (Lheureux & Chaussepied, 1995).

It was also interesting to note that Mg^{2+}-ATP changed in a similar way the fluorescence intensities of the cross-linked and non-cross-linked G-actin−S1 complexes. This demonstrated that, even in the cross-linked complexes, Mg^{2+}-ATP caused the dissociation of S1 which remained tethered to G- or F-actin. Consequently, such a tethered S1, which was cross-linked to actin residues 1−7 (Combeau et al., 1992), no longer affected the environment of the pyrene group attached to residue Cys374 (Cooper et al., 1983), though these two actin loci are close together in the actin 3-D structure (Kabsch et al., 1990).

Figure 6 summarizes the overall results of the ATPase activities determined for the different acto-S1 complexes.

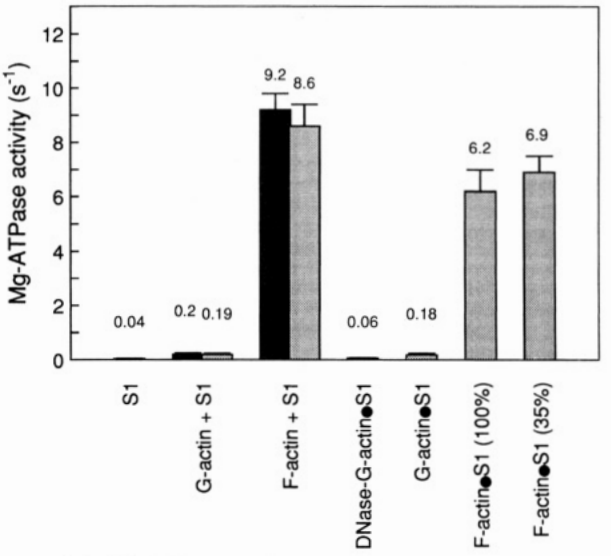

FIGURE 6: Mg^{2+}-ATPase activities of various S1-containing entities. ATPase activities were determined as described under Materials and Methods with either the colorimetric method (c.m.) or the fluorescence method (f.m.) as indicated below. Labels: S1, S1 alone (c.m.); G-actin + S1, S1 (6 μM) in the presence of G-actin (20 μM, f.m.); F-actin + S1, V_{max} obtained at infinite F-actin concentrations from three different plots of $1/v = f(1/\text{actin})$ (with 0.5 μM S1, 0−90 μM F-actin, and 1 mM Mg^{2+}-ATP, c.m. and f.m.); DNase−G-actin•S1, cross-linked ternary complex (0.8 μM, f.m.); G-actin•S1, cross-linked binary complex (0.8 μM, f.m.); F-actin•S1 (100%), cross-linked binary complex after polymerization (0.8 μM, with 1 mM Mg^{2+}-ATP, f.m. and c. m.); F-actin•S1 (35%), cross-linked F-actin−S1 complex with a cross-linking yield of 30−40% (0.8 μM, c.m.). Averaged values (with error bars) of at least three determinations are presented. The enzymatic activities were measured with native actin (solid bars) or with EDC-treated actin (stippled bars).

First of all, it is important to note that EDC treatment of actin only slightly reduced (about 10−15%) the activation of S1 by either G- or F-actin (Figure 6; compare the solid and stippled bars for native and EDC-treated actin, respectively). The larger effect of EDC reaction on the ATPase activities reported by Bertrand et al. (1989) could be explained by the fact that EDC treatment was more extensive in their case.

Second, the non-cross-linked and cross-linked binary complexes in their monomeric forms had very similar activities of 0.18−0.2 s^{-1}, corresponding to a 4−5-fold activation of the S1 ATPase activity. In contrast, the activity of the ternary DNase I−G-actin−S1(A2) complex of 0.06 s^{-1} was very close to that of purified S1 (0.04 s^{-1}).

Third, the cross-linked F-actin−S1(A2) complex exhibited a 160-fold activation with an ATPase activity value of 6.2 s^{-1}. This value was not dependent on the method used to measure the ATPase activity. Interestingly, a 30−40% cross-linked F-actin−S1(A2) complex displayed an ATPase activity of 6.9 s^{-1}, within the experimental error of the 6.2-s^{-1} value characterizing the 100% cross-linked filamentous complex. These two activities were about 20−30% lower than the maximum velocity of 8.6 s^{-1} obtained at infinite actin concentration. A lower value for cross-linked as compared to non-cross-linked filamentous complexes was also reported by other laboratories (Greene, 1984; King & Greene, 1985) and could be explained by an S1 binding in the cross-linked complex slightly weaker than it is at infinite protein concentration in the presence of nucleotides.

DISCUSSION

The present data clearly support the idea that the binding of the myosin head to two (and not only one) actin monomers is essential for the ATPase activation of the acto-S1 complex because the permanent linking of S1 to one actin monomer is not sufficient to activate the S1 ATPase activity.

It was previously shown that G-actin, even in excess over S1, could not significantly activate S1 Mg^{2+}-ATPase (Offer et al., 1972; Lheureux & Chaussepied, 1995). We demonstrated in the previous paper that this lack of activation was due to the very low affinity of G-actin for the S1−nucleotide complexes (Lheureux & Chaussepied, 1995). However, it was not clear whether the missing binding sites were really absent or simply impaired so that much higher protein concentrations—not reachable with monomeric actin in solution—should be used to restore them. Our approach to resolve this problem consisted in covalently linking the monomeric G-actin−S1 complex using the zero-length cross-linker EDC, so that the interaction between the two proteins would approximate that occurring at infinite protein concentration. The results show that the cross-linking reaction cannot restore the ATPase activation which occurs only after polymerization of the covalent complex. Therefore, the S1 binding sites needed to stabilize the actin−S1−nucleotide intermediates are absent in the actin monomer to which S1 is cross-linked. But does monomeric actin bind correctly to S1? Or in other words, could the G-actin−S1 complex be abortive? We previously found that most of the electrostatic sites characterizing the interface of the monomeric complex also exist in the filamentous state (Lheureux & Chaussepied, 1995). So the monomeric and the filamentous complexes have common interacting sites, and by consequence the acto-S1 interface of the monomeric complex can be considered as rather specific. On the other hand, the hydrophobic contacts (located on the same monomer as the electrostatic sites) and the binding of S1 to an adjacent monomer in the filament are either strongly impaired or absent in the monomeric complex. Our data strongly suggest that a full and active interaction of S1 with F-actin necessitates S1 binding to an adjacent monomer in the filament.

We also found that the cross-linked ternary DNase I−G-actin−S1 complex activates the ATPase activity neither to the level of the filamentous complex nor to the 3−5-fold activation achieved by the monomeric complex. Since DNase I does not significantly alter the binding of S1 to G-actin (Lheureux et al., 1993; Lheureux & Chaussepied, 1995), it is most probable that it prevented the formation of short oligomers which, even in a quantity as low as 2−3% of the total actin, could account for the small activation observed.

During this work we used only S1(A2) isoenzyme because the cross-linked G-actin−S1(A1) complex could not be produced in sufficient amount due to its strong tendency to polymerize spontaneously (Chaussepied & Kasprzak, 1989; Chen & Reisler, 1991; Lheureux et al., 1993). However, since most of the properties of the G-actin−S1(A2) interaction, described so far, are comparable to those of the G-actin−S1(A1) complex (Lheureux et al., 1993; Lheureux & Chaussepied, 1995), we believe that these results can be extrapolated to the two types of monomeric complexes.

Another important finding of this work is that the activation of S1 ATPase by F-actin is not dependent on the degree of saturation of the actin filament by S1. This conclusion was made possible only because we succeeded in purifying a workable amount of a covalent G-actin−S1 complex, free of contaminating non-cross-linked S1 or actin and capable of generating, after polymerization, a 100% cross-linked filamentous complex. In fact, the ATPase activity of this 100% cross-linked complex was found to be identical to that of a 30−40% cross-linked complex. One should note that it was not possible to obtain a wider range of cross-linking yield by mixing and copolymerizing G-actin and the covalent G-actin−S1 complex since G-actin polymerized at a slower rate than the complex so that the filaments obtained could not be randomly decorated.

Previous work also reported that not only the structures but also the ATPase activities of cross-linked complexes containing 1−50% cross-linked S1 were similar (Greene, 1984; King & Greene, 1985; Craig et al., 1985). Tesi et al. (1991) also proposed that, at −15°C and in the presence of 40% ethylene glycol, the degree of saturation of the non-cross-linked complex does not affect the cleavage and the P_i release steps of the acto-S1 ATPase activity. But so far nobody ever described the properties of the filaments fully saturated with cross-linked myosin heads. On the other hand, Huang et al. (1990) claimed a difference in the level of the ATPase activation depending on the cross-linking yield of the F-actin−S1 complex. However, neither the nature of the cross-linked adducts, the degree of saturation, nor the effect of the extensive EDC treatment used in their study was clearly established.

There exists a large body of evidence showing that the actin/S1 ratio changes the orientation of S1 to the filament axis (Craig et al., 1980; Andreev et al., 1993a,b) as well as the structure of the actin−S1 interface (Yamamoto, 1990; Andreev & Boredjo, 1992; Bonafé & Chaussepied, 1995). Though the kinetics of ATP binding to the complex is apparently also affected (Tesi et al., 1990), our data clearly demonstrate that the overall ATPase activity remains unaltered by the actin/S1 ratio. Andreev et al. (1993a) proposed that the S1 reorientation imposed by the actin/S1 ratio was essential for the force production process. Our data do not necessarily contradict this hypothesis, but they show that in this case the role of filament saturation on force generation should be uncoupled from the ATPase activity of the complex. Such uncoupling between ATPase activity and power stroke has also been demonstrated by several authors [see, for example, Yanagida et al. (1993) and Spudich (1995)].

In conclusion, S1 has to bind to a second monomer in order to initiate the cycle of weak to strong or of the A to R interacting states proposed by Geeves (1991). Whether the S1 contacts on the second monomer involve the top of actin subdomain 2 or actin segment 89−93 (or actin loop 18−28 in unsaturated filaments; Bonafé & Chaussepied, 1995; Rayment et al., 1993; Schröder et al., 1993) has now to be determined to figure out the exact structure of the actin−S1 interface during the overall ATP cycle.

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