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BINDING OF MYOSIN SUBFRAGMENT-1 TO F-ACTIN

O. Andreev and J. Borejdo*

Baylor Research Institute
Baylor University Medical Center
3812 Elm St., Dallas, TX 75226

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SUMMARY: During a part of the hydrolytic cycle, myosin head (S1) carries no nucleotide and binds strongly to an actin filament forming a rigor bond. At saturating concentration of S1 in rigor, S1 is well known to form 1:1 complex with actin. However, we have provided evidence that under certain conditions S1 could also form a complex with 2 actin monomers in a filament (Andreev, O.A. & Borejdo, J. (1991) Biochem. Biophys. Res. Comm. 177, 350-356). This view was recently challenged by Carlier & Didry (Carlier, M-F. & Didry, D. (1992) Biochem. Biophys. Res. Comm. 183, 970-974) who interpreted our data by suggesting that F-actin underwent a simple depolymerization and implied that, when only actin in the F-form was scored, the real stoichiometry in our experiments was 1:1. We show here that under conditions of our experiments less than 8% of actin was depolymerized. Moreover, we have repeated the experiments in the presence of phalloidin and show that under these conditions too, when S1 was added slowly to a fixed concentration of F-actin, it formed a different complex with F-actin than when it was added quickly. This confirms our original conclusion that S1 can bind actin in two different ways and shows that depolymerization of F-actin is not responsible for this finding.

It has been shown by ultracentrifugation, turbidity, fluorescence and cross-linking (1-9) that at saturation S1 formed 1:1 complex with actin. Recent data from our laboratory, however, suggests that under certain conditions S1 could also form a complex with 2 actin monomers in a filament (10). Thus, when S1 was added slowly (5-10 min between additions) to a fixed concentration of F-actin, the turbidity signal reached a plateau at a molar ratio of S1/actin of 0.5. When excess of S1 was added to such samples, the turbidity increased slowly to a second plateau corresponding to a molar ratio of $S1_{bound}$/actin of 1. When S1 was added quickly (10-20 sec between additions) then in agreement with earlier results only 1 plateau was observed corresponding to a molar ratio of $S1_{bound}$/actin of 1. The same results were obtained when binding was measured by quenching by S1 of fluorescence of F-actin labeled with pyrene (11). The simplest

*To whom correspondence should be addressed.

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interpretation of our data was that S1 initially bound to a filament in a conformation in which it attached to 1 actin monomer (A_1*S1), and that it underwent transformation (isomerization) to the second conformation (A_2*S1) in which it bound 2 actin monomers. We think that this transformation occurs only in S1's which can "lie down" on the surface of F-actin, i.e. where there is a free actin monomer next to bound S1.

However, Carlier & Didry (12) interpreted our data by suggesting that F-actin underwent a simple depolymerization. These authors showed that if concentrated actin solution was diluted by a buffer containing 50 mM KCl and no ATP and 1 hr was allowed to elapse before the measurement, only half of actin at 0.8 μM was in polymeric form. This implied that, when only actin in the F-form was scored, the real stoichiometry was 1:1. We show in this paper that under conditions of our experiments (10), only 7.8% of actin was depolymerized. Moreover, we show that when experiments were done in the presence of phalloidin (a reagent that decreases a critical concentration of F-actin to nearly 0, ref. 13), than in agreement with our earlier results (10) different binding was observed depending on the speed of addition of S1.

MATERIALS AND METHODS

Materials: ATP, Coomassie Blue, phalloidin and chymotrypsin were from Sigma (St. Louis, MO).

Solutions: F-buffer contained 50 mM KCl, 2 mM $MgCl_2$, 10 mM TRIS-HCl buffer pH 7.5, 0.2 mM $CaCl_2$ and 0.2 mM DTT. G-buffer contained 10 mM TRIS-HCl pH 7.5, 0.2 mM $CaCl_2$, 0.2 mM ATP and 0.2 mM DTT). B-buffer contained 50 mM KCl, 10 mM TRIS-HCl pH 7.5, 0.2 mM $CaCl_2$ and 0.2 mM DTT.

Proteins: Myosin, S1 and actin were prepared from rabbit skeletal muscle as described in refs 14, 15 and 16, respectively. Unless otherwise stated actin was polymerized for 2 hrs at room temperature by adding 50 mM KCl and 2 mM $MgCl_2$ and equimolar concentration of phalloidin. The concentrations of proteins were measured by absorbance, using S1 -- $A^{1\%}(280)=7.5$, G-actin -- $A^{1\%}(290)=6.3$, F-actin -- $A^{1\%}(290)=6.7$.

Light scattering: All measurements were done in an SLM 500C (Urbana, IL) spectrofluorometer at room temperature. Light scattering intensity was measured at right

angle to the incident beam with the excitation and emission wavelengths set to 450 nm and slits to 2.5 nm.

Ultracentrifugation: 1 mL samples were centrifuged for 1.5 hr in Beckman L5-75 Ultracentrifuge in SW 41 rotor at 131,000g at 20°C.

SDS-PAGE: PAGE was run as in (17) in a buffer containing 25 mM TRIS, 192 mM glycine, 0.1% SDS, in 12% polyacrylamide gels. The staining solution contained 50% methanol, 7.5% acetic acid and 0.25% Coomassie Blue. The fixing and destaining solutions contained 50% ethanol and 7.5% acetic acid. After the electrophoresis and staining the slab was dried using a Novex Gel Dryer Kit (Novex Co., San Diego, CA) and scanned by a flat bed scanner (ScanJet, Hewlett Packard, Palo Alto, CA). The ratio of actin in supernatant and in the pellet was measured from the scanned image using Image Pro Plus program (Media Cybernetics, Silver Spring, MD).

RESULTS

Carlier & Didry (12) have shown that when G-actin was depleted of ATP and was polymerized by 50 mM KCl, a dilution to 0.8 μM followed by a period of at least one hour during which diluted F-actin was allowed to stand (presumably at room temperature), resulted in a significant depolymerization of F-actin. We have repeated this experiment and, in agreement, find that when nucleotide free actin was polymerized with KCl alone or even with KCl and Mg^{2+}, a significant fraction of actin was in the G-form. In our experiment G-actin was passed through Sephadex G-50 column to remove free ATP and immediately afterwards polymerized by adding 50 mM KCl and 2 mM $MgCl_2$. After polymerization, the stock solution was diluted 62.5 times to 0.8 μM with B-buffer. Diluted samples were left standing at room temperature for 40 min before centrifugation. The results (Fig. 1K & L) show that 26% of actin was in the G-form (critical concentration=0.21 μM). Even more actin was depolymerized (41%) when actin was polymerized with KCl alone (Fig. 1M & N; critical concentration=0.32 μM). In contrast, when actin was polymerized by KCl and Mg^{2+} and the nucleotide was removed by dialysis (i.e. the samples were prepared as in ref. 10), little depolymerization occurred. This is illustrated in Fig. 1E & F. 80 μM stock solution of actin in G-buffer was polymerized by the addition of 50 mM KCl and 2 mM $MgCl_2$, polymerized actin was dialyzed overnight against B-buffer containing 2 mM $MgCl_2$ and before centrifugation diluted 100 times with B-buffer containing 0.1 mM $MgCl_2$. Densitometric measurements showed that 7.8% of

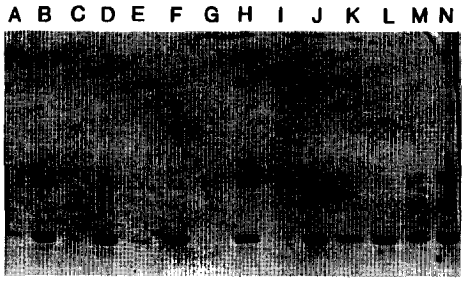

Figure 1. Centrifugation experiment to measure the critical concentration of actin. A & B: 80 μM of stock solution of G-actin in G-buffer was polymerized by the addition of 50 mM KCl. F-actin was diluted 100 times with B-buffer and centrifuged. The ratio of protein in the supernatant (A) and pellet (B) was 0.15. C & D: 80 μM stock solution of actin in G-buffer was polymerized by the addition of 50 mM KCl and 2 mM $MgCl_2$. After 1 hr polymerization at room temperature, it was diluted 100 times with B-buffer containing 0.1 mM $MgCl_2$ and centrifuged. The ratio of protein in the supernatant (C) and pellet (D) was 0.12. E & F: 80 μM stock solution of actin in G-buffer was polymerized by the addition of 50 mM KCl and 2 mM $MgCl_2$. After 1 hr polymerization at room temperature it was dialyzed overnight against B-buffer containing 2 mM $MgCl_2$. Before centrifugation it was diluted 100 times with B-buffer containing 0.1 mM $MgCl_2$. The ratio of protein in the supernatant (C) and pellet (D) was 7.8%. G & H: actin in G-buffer was passed through Sephadex G-50 column to remove free ATP. Concentration after the culumn was 50 μM. Actin was polymerized by adding 50 mM KCl. After 10 min, phalloidin at 1:1 molar ratio was added and polymerization allowed to proceed for 1 hr. F-actin was then diluted to 0.8 μM with B-buffer and centrifuged. The comparison of protein in the supernatant (G) and in the pellet pellet (H) shows that the critical concentration was less then 0.01 μM. I & J: ATP was removed, the concentration of G-actin after the column was 50 μM. Actin was polymerized by adding 50 mM KCl and 2 mM $MgCl_2$. After 10 min, phalloidin at 1:1 molar ratio was added and polymerization allowed to proceed for 1 hr. F-actin was diluted to 0.8 μM with B-buffer and centrifuged. The comparison of protein in the supernatant (I) and in the pellet pellet (J) shows that the critical concentration was less then 0.01 μM. K & L: ATP was removed, actin was polymerized by adding 50 mM KCl and 2 mM $MgCl_2$. After polymerization, the stock solution was diluted to 0.8 μM with B-buffer. Diluted samples were left standing at room temperature for 40 min before centrifugation. The ratio of protein in the supernatant (K) and pellet (L) was 0.26. M & N: ATP was removed, actin was polymerized by adding 50 mM KCl. After polymerization, the stock solution was diluted to 0.8 μM with B-buffer. Diluted samples were left standing at room temperature for 40 min before centrifugation. The ratio of protein in the supernatant (M) and pellet (N) was 0.41.

actin was in the G- form (critical concentration=0.06 μM). Similar result was obtained when ATP was not removed: 12% of actin was in the G-form (Fig. 1C & D; critical concentration=0.09 μM).

These results show that depolymerization of F-actin at low concentrations (0.8 μM) depended on the presence of Mg^{2+} and ATP in polymerizing solution. But under the conditions employed in our experiments (10) when actin was polymerized by KCl and Mg^{2+} and then dialyzed overnight against F-buffer less than 10% of actin was depolymerized. Moreover, experiments in (10) were carried out immediately after dilution, which further decreases the amount of depolymerized actin.

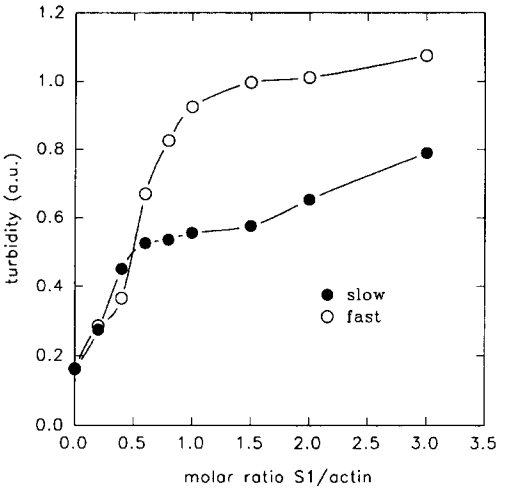

Figure 2. Effect of speed of titration on turbidity of F-actin-S1 complex. F-actin was polymerized in the presence of equimolar concentration of phaloidin, S-1 was unlabeled. Open circles: Turbidity increase in arbitrary units when increasing concentrations of S1 were added quickly (20 ± 5 sec between additions) to a fixed concentration (0.5 μM) of phalloidin-F-actin. Filled circles: Turbidity increase when increasing concentrations of S1 were added slowly (≈5 min between additions) to fixed concentration of phalloidin-F-actin. When the titrations were done slowly, the measurements were very reproducible (maximum variation of a measurement smaller than the size of the symbol). For fast titrations, especially at high MR, maximum variations were larger, but in each case less then 8% of the mean. Conditions: 0.5 μM phalloidin-F-actin, 80 mM NaCl, 10 mM TRIS-HCl,pH 7.5, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM NaN_3, 0.2 mM β-mercaptoethanol, 1 mM Na-phospate.

In agreement with (13), depolymerization could be completely inhibited by addition of equimolar concentrations of phalloidin. To remove free ATP G-actin was passed through Sephadex G-50 column equilibrated with G-buffer devoid of ATP. Immediately after the column 50 mM KCl, 2 mM $MgCl_2$ and equimolar concentration of phalloidin was added. Polymerization was allowed to proceed for 1 hr. F-actin was then diluted 62.5 times with B-buffer and centrifuged after 40 min. The supernatant and pellet are shown in Fig. 1I & J. Densitometric measurements showed that critical concentration of actin was less than 0.01 μM. The experiment was repeated on F-actin polymerized by KCl only and the results showed that F-actin was equally stable (Fig. 1G & H). We have therefore carried out all subsequent experiments on F-actin stabilized by phalloidin.

We measured increase in turbidity when S1 was added to a fixed concentration of F-actin. Fig. 2 shows that the increase in turbidity depended on the speed of titration. 0.5 μM of F-actin polymerized in the presence of equal molar concentration of phalloidin was titrated quickly (open circles, 20 ± 5 sec between additions) or slowly (filled circles, ≈5 min

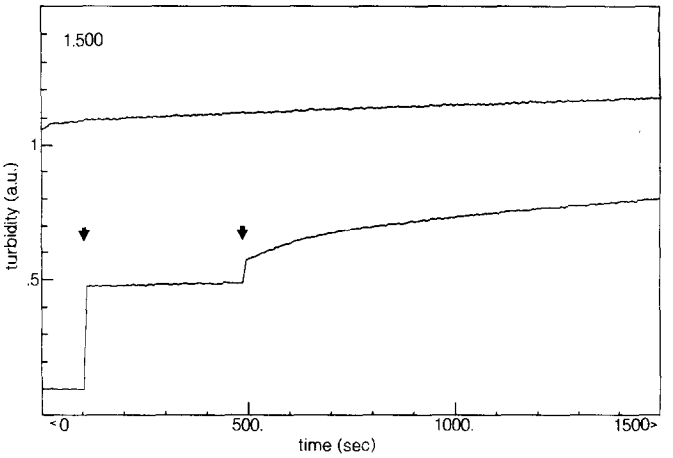

Figure 3. Transition between two types of binding. 0.5 μM F-actin alone (between time 0 and time indicated by the first arrow). During the time bracketed by the first and the second arrow, turbidity of a sample in which 0.3 μM S1 was preincubated for 20 min with 0.5 μM F-actin was measured. At a time indicated by a second arrow, additional S1 to make a molar ratio of 2 was added. Solutions:B-buffer and 0.2 mM $MgCl_2$, room temperature.

between additions of S1). The binding curves are clearly different. Thus the results are consistent with conclusions reached previously (10).

Fig. 2 shows that at a given molar ratio, depending on the speed of titration, the system can exist in two different states. The two rigor states are not equilibrium ones. Equilibrium can only be reached when actin is incubated with S1 for a long time. Each state can be transformed to other by changing molar ratio and waiting a long time. To show this, the tranformation was monitored by following the changes in turbidity. The turbidity reflects the amount of acto-S1 complexes, but not the orientation of S1 with respect to the filament axis. The transition A_2*S1 to A_1*S1 was induced by first adding S1 to molar ratio 0.6 (at first arrow in Fig. 3), and than more S1 to make molar ratio equal to 2 (second arrow). Fig. 3 shows that addition of excess S1 was accompanied by doubling of turbidity. This increase in turbidity was expected, because the amount of acto-S1 complexes was increased 2 times. The reverse, transformation A_1*S1 to A_2*S1 could not be detected by a change in turbidity, because it was not accompanied by a change in the amount of acto-S1 complexes.

DISCUSSION

In the experiments reported by Carlier and Didry (12) at least one hour was allowed to elapse between the dilution of the stock solution of F-actin and the measurement. During

this time F-actin had ample time to depolymerize. In contrast, our experiments (10) were always carried out immediately after diluting the stock solution. Further difference was that our actin was polymerized by KCl and $MgCl_2$ in the presence of 0.2 mM ATP. After polymerization it was dialyzed overnight against a buffer containing 50 mM KCl, 10 mM TRIS-HCl pH 7.5, 2 mM $MgCl_2$ and 0.2 mM DTT. F-actin was more stable when polymerized by both K^+ and Mg^+ (Fig. 1), even if the buffer with which it was diluted for the experiment did not contain Ca^+ or Mg^+. Finally, we obtained the same results when actin was polymerized in the presence of phalloidin (Fig. 2). We conclude that our original claim (10) that S1 is able to bind to F-actin in two different ways is correct.

Two further points about the results of Carlier & Didry (12) deserve a comment: the authors showed that S1 induced polymerization of G-actin present in the actin samples after dilution. If G-actin was present in our samples, rapid titrations would polymerize less actin than slow titrations (because S1 had less time to act), with the result that fast titrations would give smaller final stoichiometry of binding (S1:actin) than slow titrations. We have observed exactly opposite effect, i.e. the stoichiometry was lower for slow than for fast titration. Carlier & Didry did not carry out fast titrations and it is not possible to see what effect would fast titration have on the samples which contained 50% G-actin.

Finally, Carlier and Didry claimed that in the presence of phalloidin, saturating stoichiometry was always 1:1. But the data in their Fig. 4A shows that the stoichiometry was closer to 0.5 than 1.

The present results confirm our original conclusion that S1 and F-actin can form two different rigor complexes (10,11). The equilibrium between the two is dependent on a molar ratio of S1 to actin. We concluded that at low molar ratios, the complex where S1 bound with 2 actins was predominant (11). At higher molar ratio the complex where S1 bound to 1 actin monomer was predominant. These are two different systems, because molar ratios are different in each. It follows that their equilibrium states are different. Each system can be transformed to other by changing molar ratio. This transformation was monitored here by following the changes in turbidity. The transition A_2*S1 to A_1*S1 induced by addition of excess S1 was accompanied by doubling of turbidity (Fig. 3). This increase in turbidity was consistent with the increase in the amount of acto-S1 complexes. The reverse, transformation A_1*S1 to A_2*S1 could not be detected by a change in

turbidity, because it was not accompanied by a change in the amount of acto-S1 complexes.

We suggested (11) that the two complexes have a direct relation to intermediate states of acto-S1 during ATP hydrolysis. One attractive possibility is that during each ATP hydrolysis cycle S1 first binds to one actin (corresponding to a "weak" binding), and then to two (corresponding to a "strong" binding) actin monomers, and that this transition is accompanied by force generation. This possibility supports the idea that force is generated by orientational transition of the cross-bridge between two attached states (18).

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