

The Myosin Head Can Bind Two Actin Monomers

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SUMMARY: Force impulse is thought to be generated in muscle when myosin head (S-1), while weakly bound to actin filament, undergoes orientational change to form a strong (rigor) bond with actin. There is ample evidence that this bond involves interaction of 1 myosin head with 1 actin monomer. However, X-ray diffraction data of muscle decorated with S-1, as well as recently proposed model of the thin filaments, suggested that each S-1 molecule interacted with two actin monomers. We reinvestigated this controversy and found that the stoichiometry of acto-S-1 bond depended on the relative amounts of actin and myosin present during titrations: when increasing amounts of actin were added to a fixed amount of S-1 (i.e. when myosin heads were initially in excess over actin), the saturating stoichiometry was 1 mol of S-1 per 1 mol of actin. However, when increasing amounts of S-1 were added slowly to a fixed amount of F-actin (i.e. when actin was initially in excess over S-1), the stoichiometry at saturation was 1 mol of S-1 per 2 mols of actin. The ability of S-1 to bind either one or two actin monomers suggests a way that force could be generated during muscle contraction. © 1991 Academic Press, Inc.

The formation of a strong (rigor) complex between S-1 and F-actin is thought to be associated with force generation in muscle (1). Ultracentrifugation (2-5), turbidimetric (6) and fluorescence (7-9) titrations experiments showed that this bond involved interaction of 1 myosin head with 1 actin monomer. Cross-linking (10) of actin and S-1 with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) gave the same result. However, X-ray diffraction data of muscle decorated with S-1 suggested that S-1 molecules interacted with both strands of a filament, straddling tropomyosin in the long pitch groove (11). This conclusion was strengthened by a recently proposed model of the thin filaments (12,13).

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Original cross-linking experiments of Mornet *et al.* (14) also suggested that the stoichiometry of binding was 1 head per 2 actin monomers. In this paper we reexamined the question of the stoichiometry of rigor binding with the aim of resolving the controversy. We found that the stoichiometry depended on the relative amounts of actin and myosin present during titrations: when increasing amounts of actin were added to a fixed amount of S-1 (i.e. when myosin heads were initially in excess over actin), the saturating stoichiometry was 1 mol of S-1 per 1 mol of actin. However, when increasing amounts of S-1 were added slowly to a fixed amount of F-actin (i.e. when actin was initially in excess over S-1), the stoichiometry at saturation was 1 mol of S-1 per 2 mols of actin.

MATERIALS AND METHODS

Protein preparation and labeling: Actin and myosin subfragment-1 were prepared from rabbit skeletal muscle according to Spudich & Watt (15) and Weeds & Taylor (16), respectively. S-1 was labeled with 5-[2-((iodoacetyl)amino)ethyl]aminonaphthalene-1-sulfonic acid (1,5-IAEDANS) by incubation at 1.5 molar excess of dye for 6 hrs in ice.

Anisotropy and turbidity measurements: Anisotropy was measured in an SLM 500C fluorometer, with excitation and emission wavelengths set at 350 and 490 nm, respectively. Proteins were in 50 mM KCl, 10 mM TRIS-HCl buffer pH 7.5. Turbidity was measured in an SLM 500C fluorometer with excitation and emission wavelengths set at 490 nm. Proteins were in 50 mM KCl, 10 mM TRIS-HCl buffer pH 7.5. All measurements were carried out at 23°C.

Cross-linking: F-actin and S-1 were mixed at different molar ratios and incubated for 30 min at 22°C. 100 mM EDC was added and the sample was incubated for 10 min at 22°C. The reaction was stopped by adding 400 mM β -mercaptoethanol. Samples were run on 7.5% polyacrylamide gel in Laemmli's buffer (17), and were stained in Silver Stain (Sigma, Saint Louis, MO).

RESULTS AND DISCUSSION

We measured the anisotropy of fluorescently labeled S-1 as it bound to F-actin. In the experiment shown in Fig. 1 (open circles), we have titrated a fixed amount of S-1 labeled with 1,5-IAEDANS with increasing amounts of F-actin and measured the resulting anisotropy. As more F-actin was added, anisotropy increased, reflecting the fact that S-1 was now partly immobilized by binding to a relatively large F-actin molecule, which did not rotate on the time scale of a few nanoseconds. Since binding of S-1 to F-actin was strong, the anisotropy increased linearly and abruptly reached a plateau at 1:1 molar ratio of S-1 to actin. In this experiment S-1 was initially at a molar excess over actin.

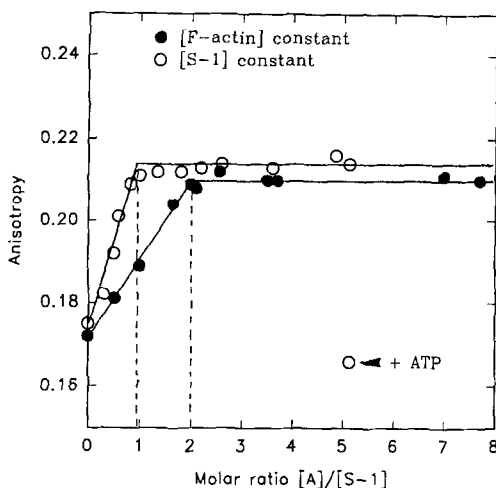


Figure 1. Increase in the anisotropy of fluorescently labeled S-1 when it binds to F-actin. Filled circles: F-actin concentration was fixed at 0.03 mg/mL and aliquotes of S-1 were added slowly to the same cuvette. Since the abscissa is the molar ratio of actin to S-1, the rightmost experimental point was obtained first, and the titrations were carried out from right to left. Open circles: S-1 concentration was fixed at 0.06 mg/mL and aliquotes of F-actin were added slowly to the same cuvette. The titration was carried out from left to right. The anisotropy after addition of 1 mM ATP is indicated by the arrow. Broken lines are projections to the abscissa of the intersection of straight lines fitted to the data in the region of increasing and constant anisotropy, respectively. Anisotropy at a molar ratio of 0 corresponds to the anisotropy of free S-1.

However, when the same experiment was carried out in reverse (filled circles), i.e. when the increasing concentrations of fluorescent S-1 were added slowly to a fixed concentration of F-actin, the anisotropy increased linearly and abruptly reached a plateau at 1:2 molar ratio of S-1 to actin. In this experiment actin was initially at a molar excess over S-1. Adding 1 mM ATP to immobilized S-1, completely reversed the rise in anisotropy (arrow). The same result was obtained when S-1 was labeled with 5-iodoacetamido-fluorescein (5-IAF) or iodoacetamido-tetramethyl-rhodamine (IATR). We have repeated these experiments using the turbidimetric method. In this method one measures the amount of light scattered by the acto-S-1 complex, and assumes a linear relationship between the amount of light scattered and the amount of the complex. These measurements have the advantage that they do not require chemical modification of S-1, and that they allow comparison with the measurements of other workers who used the same method. The results were the same as when using the anisotropy method, i.e. the S-1:actin stoichiometry was 1:1 when actin was added to S-1, and 1:2 when S-1 was added slowly to actin (not shown). Because the F-actin and K^+ ion concentrations were low and the pH was relatively high, we did not observe any S-1 induced aggregation of actin filaments (18).

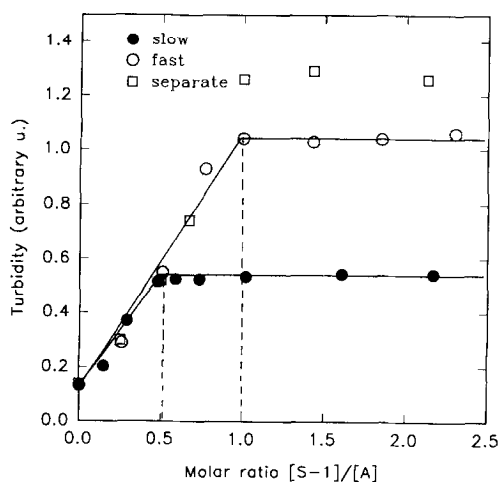


Figure 2. Turbidimetric titration of F-actin with S-1. S-1 (0-0.23 mg/mL) was added either slowly (filled circles, 600 sec between additions) or quickly (open circles, 90 sec between additions) to a fixed (0.035 mg/mL) concentration of F-actin. Broken lines are projections to the abscissa of the intersection of straight lines fitted to the data in the region of increasing and constant turbidity, respectively. The quantity 0.08 was subtracted from the turbidity values for fast titration in order to superimpose the first (no S-1) point. The turbidity of the samples that were prepared separately is shown in squares.

Even when actin is initially present at an excess over S-1, it is important to add S-1 slowly: we found that the stoichiometry depended on the speed of titration (all anisotropy experiments were carried out slowly). Fig. 2 shows that when the titration was done by adding S-1 rapidly (1.5 min between additions) to a fixed concentration of F-actin the turbidity increased linearly with [S-1] and abruptly reached a plateau at a molar ratio of S-1 to actin of 1 (open circles), as previously observed (6-9). However, when the titration was done by slowly adding S-1 to a fixed concentration of F-actin (10 min between additions), the plateau was reached at a molar ratio of S-1 to actin of 1/2 (filled circles). These results suggest that S-1 first binds with a 1:1 stoichiometry, and then slowly (over the next 5 min) relaxes to a 1:2 stoichiometry. This change of stoichiometry (and probably of the orientation of S-1 with respect to actin filament) is not reflected by a change in the turbidity, because the relaxation does not change the amount of bound S-1. Thus when the titration is done either slowly or quickly, as long as the molar ratio of S-1 to actin is less than 0.5, the amount of light scattered by the acto-S-1 complex is the same, because the mass of the scattering unit is the same (even though in each experiment S-1 presumably has a different orientation with respect to the actin filament to which it is attached).

When samples each having a different ratio of actin to S-1 were prepared separately (as in the case of ultracentrifugation experiments), the stoichiometry measured by the turbidity

method was 1:1 (squares). This is consistent with the suggestion that S-1 binds initially to 1 actin monomer. Such binding leaves space for all actin monomers to be occupied by S-1. This observation explains why earlier ultracentrifugation experiments, in which samples had to be prepared separately, clearly indicated a 1:1 stoichiometry (2-5).

When a sample exhibiting a molar ratio of S-1 to actin of 1:2 was left standing for more than 1 hr in the presence of an excess amount of unbound S-1, the complex reverted to 1:1 stoichiometry.

To summarize: only "true" titration (true in the sense that the increasing amounts of S-1 are added slowly to the same cuvette containing a fixed concentration of F-actin) produces a stoichiometry of binding of 2 actins per one S-1. When titrations are made quickly (6-9), when F-actin is added to a fixed concentration of S-1, or when true titration is not made, the stoichiometry is 1:1.

Different stoichiometries of binding must reflect different configurations of the acto-S-1 complex. When each S-1 binds to two actin monomers, it should be easier to cross-link it

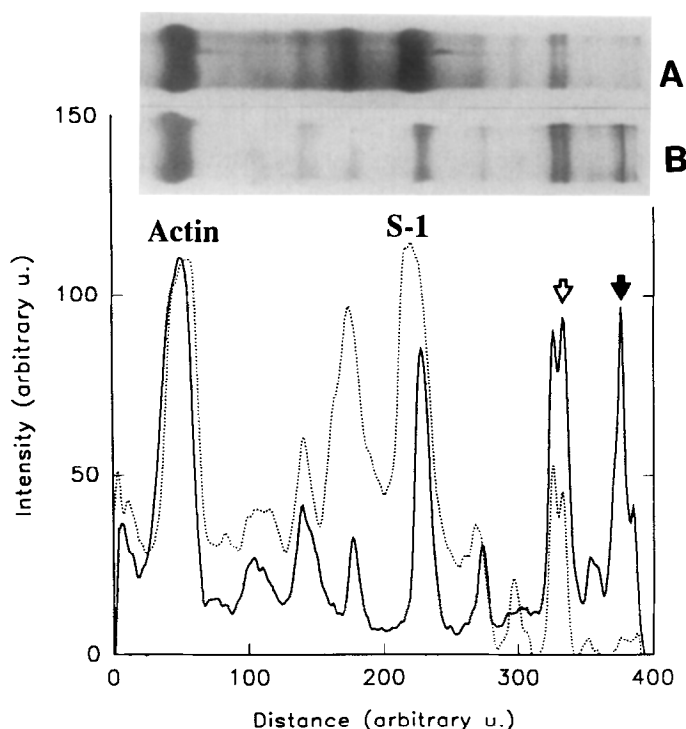


Figure 3. Cross-linking of S-1 to F-actin using different molar ratios of actin and S-1. Solid line: densitometric scan of a bottom gel shown above the scans containing 2.7 molar ratio actin:S-1 (0.31 mg/mL S-1, 0.32 mg/mL F-actin). Dotted line: densitometric scan of a top gel shown above the scans containing 2 molar ratio S-1:actin (1.7 mg/mL S-1, 0.32 mg/mL F-actin). The molecular weight was calculated from the mobility of molecular weight markers (Pharmacia, Piscataway, NJ). Solid arrow indicates 265 KDa band, open arrow indicates 175-185 KDa doublet.

to F-actin than when it binds to one actin. The cross-linked product should therefore look different depending on the stoichiometry. Fig. 3 shows that this is indeed the case. It shows the densitometric scan of the SDS-PAGE patterns of samples that have been cross-linked in different ways: the solid line is the scan of the sample that has an actin:S-1 stoichiometry of 2:1 (the SDS pattern of this sample is marked B above the scans), and the dotted line is a scan of a sample that has a stoichiometry of 1:1 (sample A). The amount of actin was the same in the two experiments. The most prominent differences between the solid and dotted lines are the absence of the 265 KDa band (indicated by solid arrow), and the smaller amount of 175-185 KDa doublet (indicated by the open arrow) when the stoichiometry is 1:1. Both of these differences show that the formation of the cross-linked products was favored when the stoichiometry was 2:1, even though in this case the absolute amount of S-1 was 5.5 times smaller (because the amount of actin was the same) and there was 2.7 times less of the acto-S-1 complex before cross-linking. The same result was obtained by Mornet *et al.* (14), who showed that the amount cross-linked product increased with increasing concentration of actin, reaching a maximal value at an actin:S-1 molar ratio near 2.

Mornet *et al.* (19) suggested that 175-185 KDa doublet could indicate 2 actins cross-linked to one S-1, and 265 KDa band could indicate actin quadruplet cross-linked to one S-1. However, measurements of Sutoh (10) indicate that the 175-185 KDa doublet is really an anomalously migrating 1:1 complex of actin and S-1. In this case the 265 KDa band must be anomalously migrating 2:1 complex of actin and S-1. We are at present checking this point.

On the basis of these results we propose that S-1 initially binds to actin with 1:1 stoichiometry. When actin is in excess and sufficient time is allowed, S-1 "lies down" on the surface of actin filament, i.e. each S-1 binds to two actin monomers. This suggestion may explain why the maximal ATPase rate and the apparent activation constant of acto-S-1 in solution is several fold different depending on whether the measurements are done keeping actin or S-1 constant (20). It also may explain the observations of Yamamoto that the binding manner between actin and the lysine-rich sequence at the junction between 50K and 20K domains of S-1 depends on the actin:S-1 molar ratio (21) and the fact that actin is necessary at 2 fold molar excess over S-1 for effective protection against the trypsin cleavage at the 50 KDa-20 KDa junction (22,23).

In physiological *rigor mortis*, since there is an excess of actin and significant time elapses from the beginning of the ATP depletion and the development of stiffness, each S-1 would

bind to two actins. During the normal ATPase cycle (24), a speculative but attractive possibility is that during each ATP hydrolysis cycle S-1 first binds to one (corresponding to "weak" binding), and then to two (corresponding to "strong" binding) actin monomers, and that this transition is accompanied by changes in orientation of S-1 with respect to thin filament. Force could be developed when the change in orientation stretches the series elasticity of the cross-bridge.

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