Regulated Crosslinked Actin Filaments and the Decoupling between Their ATPase Activity and Sliding Motility

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Troponin-tropomyosin complex from skeletal muscles was observed to regulate sliding movement of actin filaments on myosin molecules in a manner independent of their ATPase activity. When actin molecules were crosslinked with DSS (disuccinimidyl suberate), the myosin ATPase activity in the presence of the modified actin filaments complexed with both troponin and tropomyosin was only 10% less than that in the case of unmodified actin, and the ATPase activation was independent of calcium ions. In contrast, the sliding velocity of the modified actin filaments on myosin molecules decreased to zero below pCa 6.5. The present results indicate that troponin-tropomyosin complex regulates contractile movement of actomyosin systems through direct alternation of a mechanochemical property of the thin filaments, not through a decrease in the ATPase activity of the myosin molecules.

Key words: actin, calcium regulation, disuccinimidyl suberate, motility, troponin-tropomyosin complex.

Contraction of skeletal muscle is regulated by calcium ions (*1*). In the muscle cell, conformational changes of a troponin C subunit upon binding to intracellular Ca^{2+} (*2*) induce a structural transformation of a tropomyosin molecule located along an actin filament (*3*). Accordingly, the steric position of a tropomyosin molecule in relation to the filament axis of actin monomers is altered, influencing the interaction between actin molecules and myosin heads (*4-6*). Although some evidence for changes in the position of tropomyosin molecules has been reported (*7-9*), direct evidence of a relationship between the change in the position of tropomyosin and the inhibition of actomyosin interaction has not been obtained.

Observation of *in vitro* sliding of regulated actin filaments (*10, 11*) demonstrates that all types of movement including thermal motions are frozen once the concentration of calcium ion falls below the level required to maintain their active sliding movements. This may reflect weak binding ability of actin filaments in the presence of low calcium (*12-15*). It is of interest to know where tropomyosin molecules are located in relation to the actin filament in the weak-binding form.

We examined the effect of a chemical crosslinker disuccinimidyl suberate (DSS), which enhances the affinity of actin filaments toward myosin heads up to about 13 times (*16*), on the appearance of the weak-binding form of regulated actin filaments at various concentrations of calcium ion. We observed that the sliding velocity and the ATPase activity of DSS-modified actin filaments exhibit totally different behaviors as the concentration of calcium ion is varied.

MATERIALS AND METHODS

Reagents and Buffers—DSS was purchased from Nacalai Tesque (Kyoto), EGTA and HEPES were from Dojindo Chemicals (Kumamoto), and dithiothreitol (DTT) and tris(hydroxylmethyl)aminomethane (Tris) from Wako Pure Chemicals (Osaka). These and other reagents used were of special reagent grade.

HEPES buffer adjusted to pH 7.5 at 25°C was used for preparation of specimens for microscopic observation. To regulate the concentration of calcium ion, the specimens were supplemented with EGTA at the concentration of 2 mM and with various concentrations of $CaCl_2$. Estimation of the calcium ion concentration was done by using the stability constants of Sillen and Martell (*17*).

Proteins—Rabbit skeletal muscle proteins were used. Actin molecules were purified by the method of Spudich and Watt (*18*), and myosin molecules were prepared by the method of Perry (*19*). Tropomyosin complexed with troponin was obtained by the method of Ebashi and Kodama (*2*) and further purified by 2 cycles of fractionation with ammonium sulfate within the range of 25 to 37.5 g/dl. Protein concentrations were estimated by means of the biuret reaction. To prepare homogeneous samples of F-actin-troponin-tropomyosin complex, we followed the method of Ishiwata and Kondo (*20*).

Purified myosin molecules were dissolved at the concentration of 20 mg/ml in 0.6 M KCl, 10 mM DTT, 10 mM potassium phosphate buffer (pH 7.0), frozen in liquid nitrogen, stored in a deep-freeze at −80°C, and melted before use.

Crosslinking—One milligram/ml of F-actin was suspended in 50 mM KCl, 10 mM HEPES (pH 8.0), 0.5 mM ATP, and 1 mM $MgCl_2$. DSS was added to the specimen at the concentration of 2.0 mM. The solution was incubated

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for about 60 min at 20°C. The reaction was terminated by adding Tris-HCl (pH 7.5) at a final concentration of about 50 mM. The crosslinked actins were then centrifuged at 40,000 rpm at 4°C for 40 min. The pellets were suspended in 50 mM DTT and 10 mM ATP and then dialyzed against the medium for each experiment, and used without further purification. SDS-PAGE and isoelectric focusing were performed according to Laemmli (*21*) and O'Farrell *et al.* (*22*), respectively.

Microscopic Observation—The regulated filaments from either modified or unmodified actin molecules were prepared by mixing G-actin solution and troponin-tropomyosin solution with a weight ratio of 1:1 as previously described (*23*). Our *in vitro* motility assay followed the method of Harada *et al.* (*11*).

ATPase Assay—We assayed regulated actin-activated myosin ATPase under the following conditions: 0.1 mg/ml myosin, 0.2 mg/ml regulated actin, 25 mM KCl, 2 mM ATP, 2 mM $MgCl_2$, 5 mM DTT, 5 mM creatine phosphate, 40 units/ml creatine phosphokinase, 2 mM EGTA, and $CaCl_2$ at a controlled concentration. ATP hydrolysis was monitored by measuring the concentration of inorganic phosphate by the method of Ohno and Kodama (*24*).

RESULTS

Actin Crosslinking with DSS—Figure 1 shows both the SDS electrophoretic pattern and the result of isoelectric focusing. The fact that the SDS pattern of both intact and DSS-modified actin molecules remained unchanged indicates that single actin molecules were DSS-crosslinked in an intramolecular, not an intermolecular, manner with regard to their amino groups. The isoelectric focusing pattern indicated that the DSS-modified actin molecules were more acidic than the intact actin. The absence of spots corresponding to intact molecules suggests that the actin molecules we used were 100% modified.

ATPase Activity—Experimental conditions adopted for measuring the ATPase activity (see "MATERIALS AND METHODS") were similar to those in the assay for measuring the sliding velocity, with the exception that the concentration of proteins is much higher. Figure 2 displays the ATPase activation rates for both cases, normal r-FA (actin filaments complexed with troponin and tropomyosin) and r-DS-AF (DSS-modified actin filaments complexed with troponin and tropomyosin). The ATPase activation of r-FA varies in a pCa-dependent manner. In contrast, the ATPase activation of r-DS-AF was maintained at 90% of that for

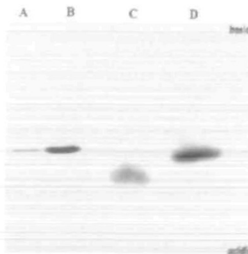

Fig. 1. **SDS-PAGE and isoelectric focusing patterns of DSS-modified actin.** The purity of DSS-crosslinked actin molecules was examined by both SDS-PAGE and isoelectric focusing. Lanes B and A indicate the effective molecular weights of intact and DSS-modified actin molecules, respectively. Both types of the molecules exhibited essentially the same molecular weight. Isoelectric points of DSS-modified and intact actin were evaluated in lanes C and D, respectively. DSS modification made the resulting actin molecules more acidic than the intact ones. Observation of a single band in lane C indicates that the modified actin preparation used in this experiment contained 100% modified molecules. Experimental conditions are described in the text.

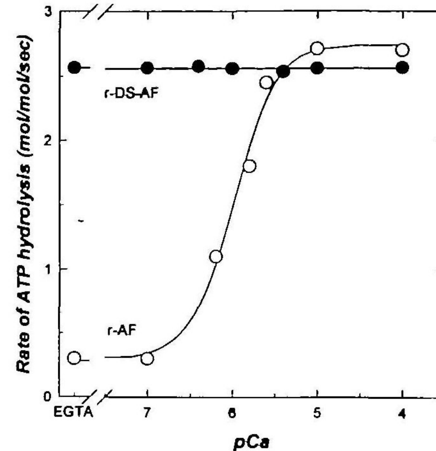

Fig. 2. **ATPase activity of DSS-actin complexed with Tn-Tm.** The rate of ATP hydrolysis was measured as a function of pCa. Conditions: 0.1 mg/ml myosin, 0.2 mg/ml regulated actin, 25 mM KCl, 2 mM ATP, 2 mM $MgCl_2$, 5 mM DTT, 5 mM creatine phosphate, 40 units/ml creatine phosphokinase, 2 mM EGTA, and calculated concentrations of $CaCl_2$. Regulated actin molecules were prepared by mixing Tn-Tm complexes with either intact or modified actin in a weight ratio of 1:1. Filled circles show r-DS-AF, and open circles, intact r-AF.

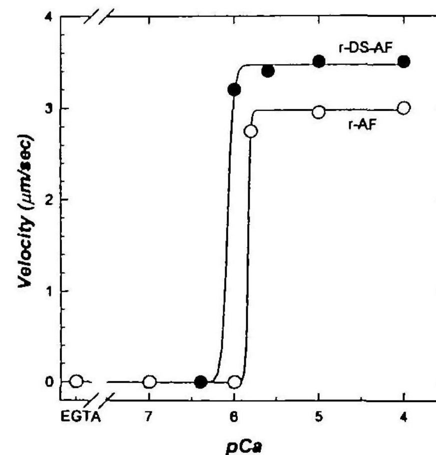

Fig. 3. **Sliding velocity of regulated actin filaments.** The velocity of sliding was measured by a conventional *in vitro* motility assay method. Filled circles show r-DS-AF and open circles, intact r-AF. Conditions: 0.2 mg/ml regulated actin, 25 mM KCl, 2 mM ATP, 2 mM $MgCl_2$, 5 mM DTT, 5 mM creatine phosphate, 40 units/ml creatine phosphokinase, 0.2 mg/ml glucose oxidase, 0.04 mg/ml catalase, 4.5 mg/ml glucose, 2 mM EGTA, and calculated concentrations of $CaCl_2$. Regulated actin molecules were prepared as described in the legend to Fig. 2. Since about 80% of filaments within a microscopic field showed motility, only moving filaments were counted for velocity measurement. Each circle indicates a value averaged over 30 independent measurements. Standard deviations were less than the size of each symbol.

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unmodified r-FA in the presence of calcium ion. The r-DS-AF ATPase activation apppeared to be independent of whether or not calcium ions were present.

Sliding Motility—Sliding movements of both normal r-AF and r-DS-AF on myosin molecules were examined under a fluorescence microscope. The sliding velocity of actin filaments is depicted in Fig. 3. The sliding velocity was observed to be regulated by calcium ion for both r-AF and r-DS-AF, contrary to the case of ATPase activation. The sliding velocity decreased to zero below pCa 6.5 for r-DS-AF, while the value of pCa at which the sliding velocity for r-AF vanished was 5.8. Although normal actin filaments exhibit thermal brownian motions below pCa 6.5, DSS-modified filaments did not display appreciable thermal motion. The presence of the pCa gap for the onset of sliding movements between r-AF and r-DS-AF was confirmed in five independent experiments.

DISCUSSION

DSS-modified actin filaments, without troponin-tropomyosin complexes, exhibit about 15 times greater affinity toward myosin subfragment-1 compared to unmodified filaments, and the velocity of sliding on a glass surface coated with myosin molecules is decreased by half (*16*). DSS is known to react with amino groups, especially with that of Lys336 in F-actin (*25*), and to form a crosslink with other amino groups at least 0.92 nm away, though the chemical properties of such DSS-modified actin monomers are yet to be clarified.

It might be argued that troponin-tropomyosin complexes could not bind to DSS-modified actin filaments in the absence of calcium ion and accordingly the ATPase activation could not be suppressed (*cf.* Fig. 2). However, the possibility of detaching troponin-tropomyosin complex from DSS-modified actin filaments in the absence of calcium ion is ruled out by the fact that the sliding movement of the filaments was strongly suppressed in the absence of calcium ion. Although increased affinity of r-DS-AF toward myosin heads might explain the enhancement of ATPase activation, this increase does not account for the calcium-ion dependent switching of the sliding movement. It might be argued that r-DS-AF could change their conformation to one different from those in the "on" and "off" states (*9*) and so could activate their myosin ATPase in almost the same way as in the "on" state.

It has already been reported that tropomyosin may sterically block the interaction of myosin heads with actin molecules (*4-7*). In contrast, we in the present study demonstrated that r-DS-AF exhibited sliding movement in a manner dependent on the concentration of calcium ion, whereas their myosin ATPase activation remained independent of calcium concentration. Troponin-tropomyosin complexes may regulate the *in vitro* sliding movement of r-DS-AF on myosin heads independently of myosin ATPase activation at low ionic strength.

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