Note

Effects of Cross-linking of SH₁ and SH₂ in Rabbit Skeletal Myosin on Heat-induced Gelation in the Presence of Actin

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Myosin solution forms a gel when it is heated under appropriate conditions.¹⁾ Yasui *et al.*^{2,3)} studied the effects of F-actin on the heat induced-gel strength of myosin at high ionic strength. They noted that gel formability was enhanced by a certain amount of F-actin in the system. This actin effect was not observed when the actin-binding site(s) of myosin was blocked by *p*-chloromercuribenzoate or when the myosin-binding site(s) of actin were blocked by trinitrophenylation. The effect also disappeared in an aging myosin-actin mixture with ATP or inorganic pyrophosphate as a dissociating agent, and appeared again after hydrolysis of ATP by myosin or the pyrophosphate by pyrophosphatase.⁴⁾ On the other hand, myosin has two sulfhydryl groups (SH₁ and SH₂) with characteristic reactivities, which are located in the 20K domain of myosin subfragment-1 (S-1) heavy chain.⁵⁾ It was reported that S-1 whose SH₁ and SH₂ were covalently cross-linked by *N,N'*-*p*-phenylenedimaleimide (*p*PDM) or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) did not bind F-actin^{6~8)} and that the binding ability of the DTNB-cross-linked S-1 to F-actin was restored after reduction of the cross-linkage.⁷⁾ In this paper, we report that the enhancement of heat-induced gelation of myosin by F-actin was completely suppressed when SH₁ and SH₂ groups of myosin were cross-linked and that the enhancement was restored after breaking of the cross-linkage.

Rabbit skeletal muscle myosin was prepared as described by Perry.⁹⁾ Rabbit skeletal muscle F-actin was purified by the method of Spudich and Watt.¹⁰⁾ ATPase activities were measured by the course of P_i liberation at 25°C. Concentrations of P_i were measured by the Fiske-Subbarow method.¹¹⁾ K⁺-EDTA ATPase was assayed in the presence of 0.5 M KCl, 2 mM EDTA, 40 mM Tris-HCl (pH 8.0), and 1 mM ATP. Ca²⁺-ATPase activity was measured in a solution containing 0.1 M KCl, 5 mM CaCl₂, 40 mM Tris-HCl (pH 8.0), and 1 mM ATP. The rigidity of reconstituted actomyosin gels was measured by

a band type viscometer as reported by Yasui *et al.*²⁾ SH₁ and SH₂ groups of myosin were cross-linked with *p*PDM as described by Sekine and Yamaguchi¹²⁾ and Katoh *et al.*⁷⁾ The degree of modification was followed with Ca²⁺-ATPase and K⁺-EDTA ATPase activities. Two mg/ml myosin was incubated with a 4-fold molar excess of *p*PDM in the presence of 0.06 M KCl, 1 mM MgCl₂, 0.5 mM ADP, and 20 mM Tris-HCl (pH 8.0) at 0°C. The cross-linking reaction was stopped by adding a 2-fold molar excess of 2-mercaptoethanol over that of *p*PDM. As reported previously,^{7,13,14)} both Ca²⁺-ATPase and K⁺-EDTA ATPase activities decreased to less than 10% of those of the control myosin after 30 min of modification. The 30-min modified myosin was collected by centrifugation after 2-fold dilution with water. The myosin was washed with 0.03 M KCl by dispersing and centrifuging to remove excess 2-mercaptoethanol. To remove contaminating myosin species of which the SH groups were not cross-linked, 3-fold molar excess of F-actin over that of myosin was added to the myosin solution containing 0.6 M KCl. The solution was stirred for 30 min and left overnight at 4°C. The solution was centrifuged at 100,000 × *g* for 120 min after 2-fold dilution with water. The three-fifth top layer of the supernatant was collected and fractionated with ammonium sulfate between 36% and 42% saturation in 0.5 M KCl and 50 mM potassium phosphate buffer (pH 7.0) to remove the remaining F-actin in the supernatant. The modified myosin was used after dialysis against 0.5 M KCl. The purity of modified myosin was examined by a polyacrylamide gel electrophoresis with sodium dodecyl sulfate by the method of Laemmli.¹⁵⁾ No actin band was observable with our treated myosin samples. SH₁ and SH₂ groups of myosin were also cross-linked with DTNB by the methods of Wells and Yount¹³⁾ and Katoh *et al.*⁷⁾ Two mg/ml myosin was incubated with a 4-fold molar excess of DTNB over that of myosin in a solution containing 0.06 M KCl, 1 mM MgCl₂, 0.5 mM ATP, and 20 mM Tris-HCl (pH 8.0) at 0°C. Ca²⁺-ATPase activity decreased to 50% of that of unmodified myosin after 72 hr followed by about 2-fold activation at the early period of the modification and K⁺-EDTA ATPase activity decreased to about 30% of that of control myosin after 72 hr. The 72-hr modified myosin was purified as described above. For reversal of the cross-linkage with DTNB, the modified myosin was incubated for 5 hr with 5 mM dithiothreitol in 0.5 M KCl and 20 mM Tris-HCl (pH 8.0) at 4°C and then dialyzed against 0.5 M KCl.

Figure 1 shows the rigidity of heat-induced gels of myosin the SH₁ and SH₂ groups of which had been cross-linked with *p*PDM, in the presence of various amount of rabbit skeletal F-actin. As reported,⁴⁾ the heat-induced gel formability of control myosin was biphasically influenced as the amount of F-actin in the system increases. The maximum rigidity of heat-induced gels of the control

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FIG. 1. Rigidity of Myosin Cross-linked between SH₁ and SH₂ Groups with *p*PDM in the Presence of F-Actin. Myosin and F-actin were mixed at a constant total protein concentration in 0.6 M KCl and stood at 0°C overnight. The rigidity of sample solutions containing 4.5 mg/ml of proteins, 0.6 M KCl, and 20 mM phosphate buffer (pH 6.0) was measured after heating at 65°C for 20 min. (○), control myosin; (●), cross-linked myosin.

myosin was found when the weight ratio of myosin to actin was about 15:1. On the other hand, the enhancing effect of F-actin on the heat-induced gelation of cross-linked myosin could not be detected at any ratios of myosin to actin tested. We also found similar results in the thermal gelation of myosin cross-linked with DTNB (Fig. 2). The enhancing effect of F-actin on the heat-induced gelation of myosin disappeared. Unlike *p*PDM which cross-linked irreversibly, the DTNB cross-linkage between SH₁ and SH₂ is reversible when it is reduced with dithiothreitol.⁷⁾ The heat-induced gel strength of reconstituted actomyosin was measured by using the reduced myosin preparation. Although the strength of rigidity was not quantitatively the same as that of the control, the enhancing effect of F-actin was clearly found to recover at the same myosin to actin weight ratio as control myosin. These results show that heat-induced gel strength of myosin is enhanced by F-actin when F-actin binds directly to myosin, but isn't enhanced when they don't bind each other and this supports our previous proposal that an actomyosin complex is formed in the myosin-actin system before gelation on heating. This is the first report showing directly that the SH groups of myosin molecules participate in the effects of F-actin on the rigidity of heat-induced myosin gels.

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FIG. 2. Rigidity of Myosin Cross-linked between SH₁ and SH₂ Groups with DTNB in the Presence of F-Actin. The experimental conditions were similar to those of Fig. 1 except that total protein concentration was kept at 5.0 mg/ml. (○), control myosin; (●), cross-linked myosin, (Δ), myosin reduced with dithiothreitol.

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