The Changes of "Myosin B" during Storage of Rabbit Muscle

Part VIII. Effect of Storage at High Ionic Strength (0.6 M KCl), pH 5.7 and 25°C on the Interaction of Myosin A and Actin

By Akihiro OKITANI and Masao FUJIMAKI

Department of Agricultural Chemistry, The University of Tokyo, Tokyo

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The denaturation process of actomyosin during storage in 0.6 M KCl at pH 5.7 and 25°C was studied with regard to changes in the binding ability of myosin A and actin.

When F-actin was stored alone, it lost the ability to bind with myosin A in parallel to the decrease in the ATPase activating ability. When myosin A was stored alone for 3 hr, it maintained 60% of the initial ability to bind with F-actin, even if it lost 85% of the initial ATPase activity. Thus, the ATPase-less myosin A forms a myosin A-actin complex when it is mixed with intact F-actin. F-Actin was not stabilized in this modified myosin A-actin complex. This complex was dissociated with ATP into ATPase-less myosin A and intact F-actin.

The modified myosin A-actin complex did not occur in the stored actomyosin. During the storage of actomyosin, the interaction between myosin A and F-actin did not break and became less dissociable by ATP. This feature is in contrast with the one observed previously on the actomyosin stored in 0.6 M KCl at pH 5.7 and 3°C.

In order to contribute to elucidation of a resolution process of muscle rigor at a relatively high temperature, changes in myosin A and the actin component when myosin B was stored in 0.6 M KCl at pH 5.7 and 25°C was studied in the present work. In a preceding paper¹⁾ were described the changes in ATPase activity, turbidity and solubility of actomyosin. In this paper are reported the results on the measurement of the binding ability.

Insted of myosin B (natural actomyosin), synthetic actomyosin was used as sample preparations to make data analysis simple.

MATERIALS AND METHODS

Preparation of proteins. Myosin A and F-actin were prepared from minced rabbit muscles (*Longissimus dorsi*) as described in the preceding paper.¹⁾ Actomyosin was obtained by mixing myosin A and F-actin in the rate of two to one by weight.

Storage of proteins. Proteins were stored as decribed in the preceding paper.¹⁾

Determination of ATPase activity of isolated myosin A or myosin A in actomyosin. It was conducted as in the preceding paper.¹⁾

Determination of ATPase activating ability of isolated F-actin or F-actin in stored actomyosin. It was conducted as in the preceding paper.¹⁾

1) A. Okitani and M. Fujimaki, *Agr. Biol. Chem.*, **34**, 1716 (1970).

Determination of actomyosin forming ability of stored myosin A. Varying amounts of myosin A were mixed with a relatively large amount of F-actin (0.6 mg) and the mixture was left for 1 hr at room temperature and pH 7.2 in 0.038 M KCl, where the actomyosin formed was precipitated and the remainder of F-actin was soluble. After the centrifugation of the mixture at $2500\times g$ for 10 min, the protein content of the supernatant was determined with optical density at $280\text{ m}\mu$. As shown in Fig. 1, actomyosin was found

FIG. 1. Actomyosin Formation of F-Actin with Varying Amounts of Myosin A.

F-Actin (0.6 mg/ml) was mixed with myosin A (0~0.38 mg/ml) in 0.038 M KCl-0.02 M tris buffer (pH 7.2) and the mixture was left for 1 hr at room temperature. After the centrifugation of the mixture at $2500\times g$ for 10 min, the optical density of the supernatant at $280\text{ m}\mu$ was measured.

to be formed in proportion to the amount of added myosin A when the amount of myosin A is less than 0.25 mg under the condition used here, the stored myosin which had lost the actomyosin forming ability was not contained in the supernatant. Accordingly, the actomyosin forming ability of the stored myosin A was determined by the measurement of the supernatant when 0.23 mg of the stored myosin A was mixed with 0.6 mg of intact F-actin.

Determination of actomyosin forming ability of stored F-actin. Varying amounts of F-actin were mixed with a relatively large amount of myosin A (0.5 mg) and the mixture was left for 16 hr at 3°C and pH 7.2 in

0.1 M KCl, where the actomyosin formed was precipitated and the remainder of myosin A was soluble. After the centrifugation of the mixture at $5000\times g$ for 15 min, the protein content of the supernatant was determined with UV absorption. The supernatant was so turbid that its optical density at $260\text{ m}\mu$ was measured after adding 2.2% (final) NaOH for clarification. As shown in Fig. 2 the result indicates that

FIG. 2. Actomyosin Formation of Myosin A with Varying Amounts of F-Actin.

Myosin A (0.5 mg/ml) was mixed with F-actin (0~0.08 mg/ml) in 0.1 M KCl-0.02 M tris buffer (pH 7.2) and the mixture was left for 16 hr at 3°C . After the centrifugation of the mixture at $5000\times g$ for 15 min, the optical density of the supernatant at $260\text{ m}\mu$ was measured in 2.2% NaOH (*cf.* Methods).

actomyosin is formed in proportion to the amount of added F-actin when the amount of F-actin is less than 0.05 mg. Under the condition used here, the stored F-actin which had lost the actomyosin forming ability was not contained in the supernatant. Accordingly, the actomyosin forming ability of the stored F-actin was determined by the measurement of the supernatant when 0.05 mg of the stored F-actin was mixed with 0.5 mg of intact myosin A.

Sedimentation analysis and determination of protein concentration. It was conducted as described in the preceding paper.¹¹ The sedimentation constants obtained were corrected to the viscosity of water at 20°C . The values extrapolated to the protein concentration of zero were not determined. Thus, all the values shown in the present paper were the observed ones (s_{20}) and were used only for qualitative comparison.

FIG. 3. Sedimentation Patterns of Stored Actomyosin with and without ATP.

- (a) 0 hr-stored actomyosin. (d) (a)+ATP.
 (b) 24 hr-stored actomyosin. (e) (b)+ATP.
 (c) 48 hr-stored actomyosin. (f) (c)+ATP.

Actomyosin of 5 mg/ml was stored in 0.6 M KCl and 33 mM tris-maleate buffer solution pH 5.7 and at 25°C. Sedimentation analysis was carried out under the following condition; 3.75 mg/ml actomyosin, 0.6 M KCl, 25 mM tris-maleate, pH 7.2, 23~26°C and 55,430 rpm. 2 mM ATP and 2 mM MgCl₂, when added. Times after reaching the top speed is shown below each diagram. The observed values for the sedimentation constant (s_{20}) are shown below each peak.

RESULTS

Sedimentation behaviour of stored actomyosin and stored myosin A

When actomyosin was stored in 0.6 M KCl at pH 5.7 and 25°C, the sedimentation pattern changed as shown in Fig. 3. Actomyosin

showed only one peak, its observed sedimentation constant being 22S before storage (Fig. 3a). On 24 hr-storage of the actomyosin were observed three peaks, their sedimentation constants being 7.9S, 40S and 47S, respectively (Fig. 3b). The 40S and 47S components seem to be the aggregated products of the 22S com-

FIG. 4. Sedimentation Patterns of Stored Myosin A.

Myosin A of 5 mg/ml was stored in 0.6 M KCl and 33 mM tris-maleate buffer solution, pH 5.7 and at 25°C for 0 hr (a), 2/3 hr (b), 4 2/3 hr (c), 24 hr (d) or 74 hr (e). Sedimentation analysis was carried out under the following condition; 2.3 mg/ml myosin A, 0.58 M KCl, 16 mM tris-maleate, pH 7.2, 10~12°C and 55,430 rpm. Photographs were taken 50 min after reaching the top speed except the case of (e) (25 min). The observed values for the sedimentation constant (s_{20}) are shown below each peak.

ponent of fresh actomyosin, and these two components seem to become 62S and 64S ones of 48 hr-stored actomyosin through the aggregation process (Fig. 3c). The aggregation process continued during 72 hr-storage (not shown in Figure).

When myosin A was stored alone in 0.6 M KCl at pH 5.7 and 25°C, the sedimentation pattern changed as shown in Fig. 4. Myosin A showed only one peak, its observed sedimentation constant being 4.3S before storage. The aggregation was observed to proceed during the storage of myosin A. This aggregation seems to be a side-to-side one as reported by many workers^{2,3)} for the denaturation of myosin A. On 2/3 hr-storage of myosin A were observed three peaks, their sedimentation constants were 5.1S, 6.1S and 6.6S, respectively, and on 4 2/3 hr-storage only one peak of 8.1S was recognized. The 24 hr-stored myosin A showed 10.5S component and the 74 hr-stored one also showed only 10.5S component. It is, therefore, suggested that 10.5S component is a final product of myosin A

aggregation under the condition used here.

The above-mentioned results on myosin A suggest that the minor 7.9S component of 24 hr-stored actomyosin (Fig. 3b) is a myosin A aggregate released from actomyosin. Moreover, the 38S component in 48 hr-stored actomyosin (Fig. 3c) is indicated not to be a myosin A aggregate. This component is therefore suggested to be a fragment derived from an actomyosin aggregate.

When F-actin was stored alone in 0.6 M KCl at pH 5.7 and 25°C, the aggregation proceeded endlessly and consequently the solubility was lost completely as shown previously.¹⁾ Accordingly, it is suggested that the endlessness observed on the aggregation process of actomyosin is attributed to rather the property of F-actin component than that of myosin A component.

Actomyosin forming ability of stored F-actin and myosin A

When F-actin was stored in 0.6 M KCl at pH 5.7 and 25°C, the actomyosin forming ability and the ATPase activating ability changed as shown in Fig. 5. The result shows that the actomyosin forming ability decreases in parallel to the decrease of the ATPase

2) S. Lowey and A. Holtzer, *J. Am. Chem. Soc.*, **81**, 1378 (1959).

3) P. Johnson and J. Rowe, *Biochim. Biophys. Acta*, **53**, 343 (1961).

Fig. 5. ATPase Activating Ability and Actomyosin Forming Ability of Stored F-Actin.

F-Actin of 0.42 mg/ml was stored in 0.6 M KCl and 33 mM tris-maleate buffer solution, pH 5.7 and at 25°C. ATPase activating ability (●) was calibrated from the value of Mg-enhanced ATPase activity of the mixture of the stored F-actin with intact myosin A. Mg-enhanced ATPase was assayed in the following mixture at 25°C and pH 7.2 for 5 min; 6 μ g/ml F-actin, 125 μ g/ml intact myosin A, 0.026 M KCl, 6 mM tris, 1 mM MgCl_2 and 1 mM ATP. Actomyosin forming ability (○) was determined for the mixture of 0.05 mg/ml F-actin with 0.5 mg/ml intact myosin A under the same condition as in Fig. 2.

activating ability. Therefore, it is suggested that F-Actin which has lost the ATPase activating ability can not combine with myosin A.

When myosin A was stored in 0.6 M KCl at pH 5.7 and 25°C, the actomyosin forming ability and the ATPase activity changed as shown in Fig. 6. Thus, it is shown that the actomyosin forming ability decreases very slowly as compared with the ATPase activity. After 3 hr-storage, the actomyosin forming ability decreased only by 40% of the initial value, while the ATPase activity decreased by 85%. However, the actomyosin forming ability was determined here at such a low salt concentration as 0.038 M KCl.

The actomyosin forming ability of stored myosin A was also investigated at such a high

Fig. 6. ATPase Activity and Actomyosin Forming Ability of Stored Myosin A.

Myosin A of 5 mg/ml was stored in 0.6 M KCl and 33 mM tris-maleate buffer solution, pH 5.7 and at 25°C. ATPase activity (●) was assayed in the following mixture at 25°C and pH 7.2 for 5 min; 0.75 mg/ml myosin A, 0.6 M KCl, 10 mM Tris, 1 mM CaCl_2 and 1 mM ATP. Actomyosin forming ability (○) was determined for the mixture of 0.23 mg/ml myosin A with 0.6 mg/ml intact F-actin under the same condition as in Fig. 1.

salt concentration as 0.6 M KCl by sedimentation analysis as shown in Fig. 7. The 1 hr-stored myosin A with 50% of the initial ATPase activity contained a large amount of aggregates with two peaks of 6.1S and 6.9S. When this myosin A was mixed with intact F-actin, the sedimentation pattern showed two major peaks of 29S and 38S. The result indicates that most of myosin A aggregates combine with F-actin and form a modified myosin A-actin complex. A minor peak of 10.5S observed in Fig. 7b is assumed to be a myosin A aggregate which has lost the actomyosin forming ability.

It is interesting to know how ATPase-less myosin A combines with F-actin. From this point of view, it was investigated whether or not the bond in the modified myosin A-actin complex was broken with ATP. When ATP was added to this complex the sedimentation pattern became similar to that of 1 hr-stored myosin A (Compare Fig. 7c with Fig. 7a). It is, therefore, concluded that the modified

FIG. 7. Sedimentation Patterns of a Mixture of Stored Myosin A and Intact F-Actin.

- (a) 1 hr-stored myosin A.
 (b) 1 hr-stored myosin A + F-actin.
 (c) 1 hr-stored myosin A + F-actin + ATP + MgCl_2 .

Myosin A of 8.3 mg/ml was stored in 0.6 M KCl and 33 mM tris-maleate buffer solution pH 5.7 and at 25°C. Sedimentation analysis was carried out under the following condition; 2.5 mg/ml myosin A, 1.25 mg/ml intact F-actin when added, 0.6 M KCl, 25 mM tris-maleate, 2 mM ATP and MgCl_2 when added, pH 7.2, 25°C and 55,430 rpm. Times after reaching the top speed are shown below each diagram. The observed values for the sedimentation constant (s_{20}) are shown below each peak.

myosin A-actin complex is dissociated with ATP as similarly as normal actomyosin.

Stabilizing effect of ATPase-less myosin A on F-actin

Intact myosin A stabilizes F-actin in 0.6 M KCl at pH 5.7 and 25°C as described in the preceding paper.¹¹ To know whether or not ATPase-less myosin A also stabilizes F-actin, two kinds of mixtures were prepared. One mixture consisted of 1 mg of F-actin and 2 mg of stored myosin A whose ATPase had been already inactivated to 16% of the initial value,

but whose actomyosin forming ability remained 60% of the initial value. Another mixture consisted of 1 mg of F-actin and 0.32 mg of intact myosin A, the total ATPase activity of which was equal to that of 2 mg of the stored myosin A used above. When these mixtures were stored in 0.6 M KCl at pH 5.7 and 25°C for 2 hr, the ATPase activating ability of F-actin component was measured by the same method as described in the preceding paper,¹¹ and the result is shown in Table I. The value obtained for the former was 43.3% of the initial one, and was almost the same as the

TABLE I. ATPASE ACTIVATING ABILITY OF F-ACTIN AFTER STORAGE WITH ATPASE-LESS MYOSIN A

Additives to 1 mg F-actin	ATPase activating ability of F-actin after 2 hr-storage ^{a)} (% initial value)
none	17.3
2 mg myosin A whose ATPase was inactivated to 16% initial ^{b)}	43.3
0.32 mg intact myosin A	42.9

ATPase activating ability was determined by the measurement of Mg-enhanced ATPase activity of the stored mixture controlled to contain 6 $\mu\text{g/ml}$ actin and 125 $\mu\text{g/ml}$ intact myosin A as in Fig. 5.

^{a)} Storage condition; 0.42 mg/ml F-actin, myosin A at an appropriate concentration when added, 0.6 M KCl, 33 mM tris-maleate, pH 5.7 and 25°C.

^{b)} Inactivation was performed by the storage of 1.67 mg/ml myosin A in 0.6 M KCl and 33 mM tris-maleate at pH 5.7 and 25°C for 3 hr.

FIG. 8. Low Temperature Effect on Aggregated Actomyosin.

(a) Aggregated actomyosin.

(b) Aggregated actomyosin stored in 0.6 M KCl and 33 mM tris-maleate at pH 5.7 and 0°C for 16 hr.

(c) Aggregated actomyosin with ATP.

Aggregated actomyosin was obtained by the storage of intact actomyosin in 0.6 M KCl and 33 mM tris-maleate buffer solution pH 5.7 and at 25°C for 72 hr. Sedimentation analysis was carried out under the following condition; 3.75 mg/ml actomyosin, 0.6 M KCl, 25 mM tris-maleate, 2 mM ATP and MgCl_2 when added, pH 7.2, 23~25°C and 55,430 rpm. Times after reaching the top speed are shown below each diagram. The observed values for the sedimentation constant (s_{20}) are shown below each peak.

one of the latter 42.9%. This result shows that the amount of F-actin remaining intact corresponded to that of intact myosin A contained initially in these mixtures. Thus, it is found that F-actin is stabilized by ATPase-active myosin A, but not by ATPase-less myosin A.

Sensitivity of stored actomyosin to ATP

It was interesting to know whether or not the above-mentioned modified myosin A-actin complex occurred on the storage of actomyosin. As shown in Fig. 3, when ATP was added to stored actomyosin, the released myosin A was inspected with regard to the presence of a dimer or a trimer by sedimentation analysis. When ATP was added to 24 hr- or 48 hr-stored actomyosin, neither a dimer nor a trimer of myosin A was observed except a monomer of 5S (Figs. 3e and 3f). Accordingly, it is confirmed that the modified myosin A-actin complex does not occur in stored actomyosin.

Moreover, Fig. 3 shows that the amount of myosin A which was released by ATP decreased with the progress of the storage of

actomyosin. It is, therefore, found that the interaction between myosin A and F-actin became insensitive to ATP on storage.

Low temperature effect on aggregated actomyosin

In our previous paper⁴⁾ was reported that the irreversible dissociation of actomyosin into myosin A and actin was caused by storing in 0.6 M KCl at pH 5.7 and 0~3°C. When the aggregated actomyosin which had been stored in 0.6 M KCl at pH 5.7 and 25°C for 72 hr was stored again in 0.6 M KCl at pH 5.7 and 0°C for 16 hr, the sedimentation pattern changed as shown in Fig. 8. The result shows that the aggregated actomyosin transforms to a somewhat low molecular one by low temperature storage (compare Fig. 8a with Fig. 8b) and that the effect of low temperature was similar to that of ATP (Fig. 8c).

DISCUSSION

By sedimentation analysis and the selective

4) A. Okitani, O. Takagi and M. Fujimaki, *Agr. Biol. Chem.*, **31**, 939 (1967).

precipitation method in the present works it was found that while myosin A loses ATPase activity by thermal treatment at a low pH value, it keeps the actomyosin forming ability. Bárány *et al.*⁵⁾ reported that by ultrasonic treatment on myosin A, its ATPase activity decreased more rapidly than its actomyosin forming ability. From the result of Kalder *et al.*,⁶⁾ by ultraviolet irradiation, myosin A lost its ATPase activity more rapidly than the actomyosin forming ability. It is, therefore, assumed that the active site of the ATPase is more susceptible to various treatments than the binding site.

The present results on the modified myosin A-actin complex give some informations on the binding site of ATPase-less myosin A. Because this complex shows specificity to ATP, it is not probable that F-actin is combined with a non-specific site of ATPase-less myosin A. Thus, the following two schemes are proposed. (1) Myosin A has at least two binding sites.⁷⁾ When both of the sites are intact, F-actin is stabilized. The first binding site degrades with the active site of ATPase more rapidly than the second one. Through the second binding site myosin A combines with F-actin when the first site is inactivated. (2) The binding site degrades with the active site of ATPase. The myosin A molecule which has lost ATPase activity and the binding ability aggregates with a intact myosin A molecule. Thus, the aggregated myosin A combines with F-actin through the binding site of the intact myosin A molecule. Either of the proposed schemes can explain the result that the stabilizing effect of stored myosin A on F-actin is proportional to remaining ATPase activity. Further studies are necessary to know which scheme fits to the modified myosin A-actin

complex.

It is expected that the modified myosin A-actin complex is formed in stored actomyosin, assuming that the ATPase of the myosin A component becomes inactive more rapidly than the actomyosin forming ability of the F-actin component. However, the fact is that the modified myosin A-actin complex does not occur in stored actomyosin as shown by sedimentation analysis, being consistent with the previous result¹⁾ that the ATPase activity of myosin A became inactive at the same rate as the ATPase activating ability of F-actin during the storage of actomyosin.

It is shown by sedimentation analysis that actomyosin does not dissociate but aggregates on the storage in 0.6M KCl at pH 5.7 and 25°C. This result is consistent with those on the turbidity and the solubility reported in the preceding paper.¹⁾

The fact that actomyosin became less dissociable by ATP suggests that the interaction between myosin A and F-actin does not become less strong during the storage in 0.6M KCl at pH 5.7 and 25°C. This feature is in contrast with the one observed previously⁴⁾ on actomyosin stored in 0.6M KCl at pH 5.7 and 3°C. Accordingly, it is unlikely that in the rigor resolution process at a relatively high temperature, a low pH value is a factor which brings about the weakening of the interaction between myosin A and F-actin. Therefore, other factors need to be researched. From this point of view, the effect of cathepsin D on actomyosin system is now being studied, and a part of the results has already been reported.⁸⁾

It was found that the aggregated actomyosin is transformed to a low molecular one by incubation in 0.6M KCl at pH 5.7 and 0°C. Such a condition as mentioned above may be useful for producing emulsion-type meat products from the muscle of poor quality in

5) M. Bárány, K. Bárány and H. Oppenheimer, *Nature*, **199**, 694 (1963).

6) G. Kaldor, J. Giltin, F. Westley and B. W. Volk, *Biochemistry*, **3**, 1137 (1964).

7) H. S. Slayter and S. Lowey, *Proc. Natl. Acad. Sci.*, **58**, 1611 (1967).

8) A. Suzuki, A. Okitani and M. Fujimaki, *Agr. Biol. Chem.*, **33**, 1723 (1969).

hydration.

The results of the preceding¹⁾ and the present works may show the way to investigate the denaturation process of contractile proteins in the pale, exudative and soft muscle

(PES muscle⁹⁾) occurring under the physiological condition of a high temperature and a low pH.

⁹⁾ R. N. Sayre and E. J. Briskey, *J. Food Sci.*, **28**, 675 (1963).