BBA 45800

STRUCTURAL CHANGES IN MYOSIN B DURING THE PROCESS OF SUPERPRECIPITATION

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(Received December 3rd, 1968)

SUMMARY

Superprecipitation of myosin B was studied, using the techniques of light scattering (turbidity and polarization ratio) and electron microscopy. With a dilute myosin B suspension (<0.05 mg/ml) in 5 mM KCl containing 20 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$, the rate of turbidity change caused by addition of ATP was found to follow first order kinetics with respect to the myosin B concentration. The measurement of the polarization ratio, ρ, as a function of scattering angle θ, showed that a myosin B suspension, with or without ATP, gives a ρ–θ relationship, indicating a minimum value at 90°, but that the ρ value in the presence of ATP is substantially higher than that in the absence of ATP.

The electron micrographs revealed that myosin B in 5 mM KCl assumes the "arrow head" structures. Immediately after addition of ATP the "arrow heads" tended to disappear and the dense clusters became attached to the thin filaments. From these results, three possible steps involved in superprecipitation are discussed.

INTRODUCTION

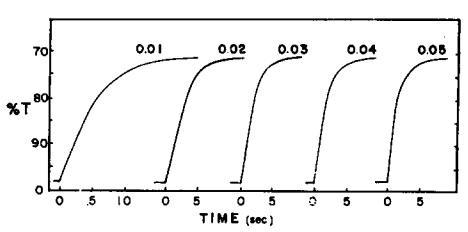
From all evidence available at present, the interaction between actin and myosin is considered to provide the physico-chemical basis of muscle contraction. In a physiologically organized system such as myofibrils, actin and myosin are separately oriented in the fibrous structures and their interaction is well described morphologically by the "sliding model" (ref. 1). Because of the clearness of this model, current biochemical studies on the actin–myosin interaction have been focused on the dissociation–association phenomenon of actomyosin[2,3]. Superprecipitation of actomyosin, however, is apparently different from a simple association reaction of actin and myosin, in that actomyosin gel becomes highly constricted particles[4]. This paper reports a study of superprecipitation, using the techniques of rate determination of turbidity change, light scattering measurement, and electron microscopy. Superprecipitation observed under the condition used here consisted of at least three steps: (1) the resolution of the "arrow head" structure, (2) the attachment of dense clusters on thin filaments and (3) the formation of large aggregates. The turbidity change measured in this work was deduced to represent the change in refractive index of myosin B, which probably corresponds to step (2) where the dense clusters are formed.

MATERIALS AND METHODS

Myosin B was prepared from rabbit skeletal muscle using the procedure of EBASHI AND EBASHI[5], and the preparation was washed with water according to the method devised by PERRY *et al.*[6]. The rate of turbidity change was determined in a Zeiss PMQ II spectrophotometer and recorded on a Servo Riter II (Texas Instruments) at a chart speed of 8 inch/min. The reaction mixture was stirred by a magnetic stirrer installed in the cell compartment, and the optimal speed was found empirically. Some fluctuation of the stirring speed did not affect the results but the stirring had to be continued until the end of the reaction. The measurement of light intensity scattered by myosin B suspension as a function of scattering angle was performed in a Brice–Phoenix photometer, which was equipped with a magnetic stirrer under a Phoenix C-101 scattering cell. The polarization ratio $\rho_\theta = H_\theta/V_\theta$ was measured, using the polaroid sheets supplied with the instrument[7]. Here H_θ is the scattering ratio for the horizontally polarized incident light, and V_θ refers to the same quantity except that the component of light is vertically polarized. The electron micrographs were taken in a Philips EM 200 electron microscope using the negative stain (phosphotungstic acid) described by BRENNER AND HORNE[8]. For the observation of superprecipitation, myosin B was diluted to a final concentration of less than 0.05 mg/ml in 5 mM KCl solution containing 1 mM $MgCl_2$, and 10 mM Tris–HCl (pH 7.4). To initiate the reaction, ATP was added to an appropriate final concentration (0.01–1.0 mM).

RESULTS

Superprecipitation is detectable either by observing the changes in viscosity or turbidity of an actomyosin suspension or in the volume of gel phase when packed under a centrifugal force. Although the measurement of turbidity in a spectrophotometer is affected by the optical arrangement of the instrument used[9], changes in optical characteristics of actomyosin provide a means with which to follow the time course of superprecipitation. The rate determined by the turbidity method suggests that under certain ionic conditions, superprecipitation occurs without dissociation[10,11].



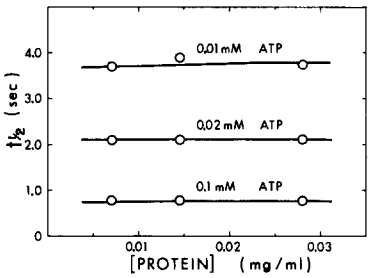
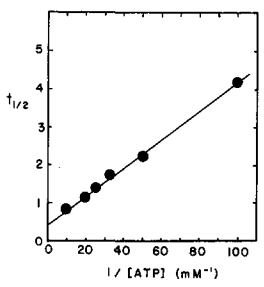


Fig. 1. Time course of superprecipitation. Relative intensity of transmitted light (545 nm) through the myosin B suspension was recorded as shown and the absorbance was calculated to determine the value of $t_{1/2}$, as defined in the text. At zero time ATP was added to a myosin B suspension (0.028 mg/ml) in 5 mM KCl + 0.02 M Tris–HCl (pH 7.4) + 5 mM $MgCl_2$. The concentrations (mM) of added ATP are indicated on top of the curves. Reaction temp., 25°.

Fig. 2. Plots of $t_{1/2}$ *vs.* the concentration of myosin B. The $t_{1/2}$ values were calculated from the experiments as shown in Fig. 1. The ATP concentrations are indicated along the lines drawn through the experimental points.

From the experiments represented in Fig. 1, the time which is required to give 50% of total turbidity change, $t_{1/2}$, can be measured. The values of $t_{1/2}$ depend on the concentration of ATP when the other ion concentrations are fixed, but are independent of the concentration (<0.05 mg/ml) of protein at fixed ATP concentration (Fig. 2). The results can be plotted to show that the rate *vs.* ATP concentration follows the Michaelis–Menten kinetics (Fig. 3), since the turbidity change is first order in myosin B concentrations. These observations would suggest that myosin B consists of a number of units, all of which undergo a certain physical change independent of each other.



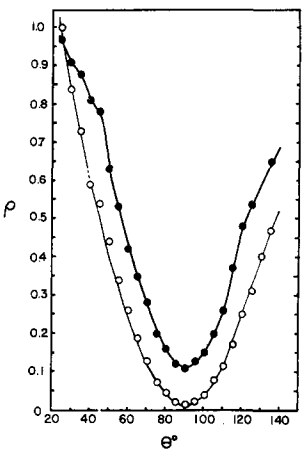


Fig. 3. Relationship of $t_{1/2}$ and 1/[ATP]. If the rate of superprecipitation follows the first order rate equation with respect to myosin B, $t_{1/2}$ should be inversely proportional to the first order rate constant. Then, $t_{1/2}$ *vs.* 1/[ATP] represents the double reciprocal plot.

Fig. 4. Polarization ratio, ρ, as a function of scattering angle, θ. Myosin B (0.03 mg/ml) was suspended in 5 mM KCl, 10 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$. ○—○, without ATP; ●—●, with 0.1 mM ATP.

Since the change in turbidity can be brought about by altering any combination of the following parameters of gel particles such as size, shape, and density (refractive index of gel particles), some other technique to characterize the physical change reflected by turbidity is required. The measurement of scattered light intensities as a function of scattering angle is a sensitive method to distinguish the size and shape of suspended particles, but theoretical interpretation of such data with irregularly shaped particles is difficult. To determine the size distribution of synthetic spherical polymers with variable refractive indices, KERKER *et al.*[12] formulated a method in which the measurement of polarization ratios is utilized. This method, based on the theory of MIE[13] for light scattering of spherical particles, can be applied, at least for qualitative purposes, to irregularly shaped particles. The plots of polarization ratios against the scattering angle are shown in Fig. 4. The curves obtained with myosin B before and after the addition of ATP demonstrate the minima at about 90°. The value of the polarization ratio differs significantly between the two cases. The ρ–θ relationship is expected to show a shift in the angles (M) at which the minima and/or maxima of ρ values are observed, when the size distribution of the particles changes[12].

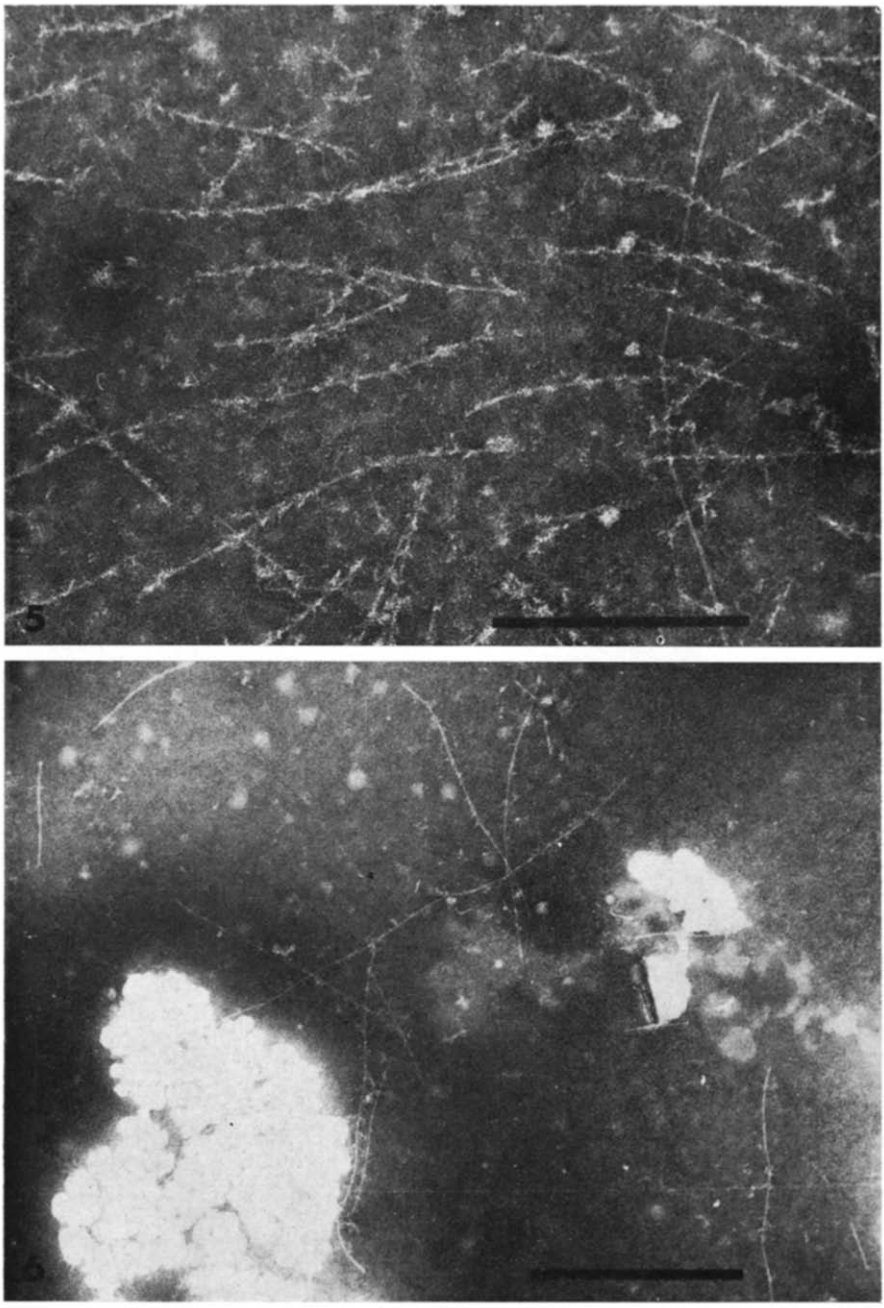
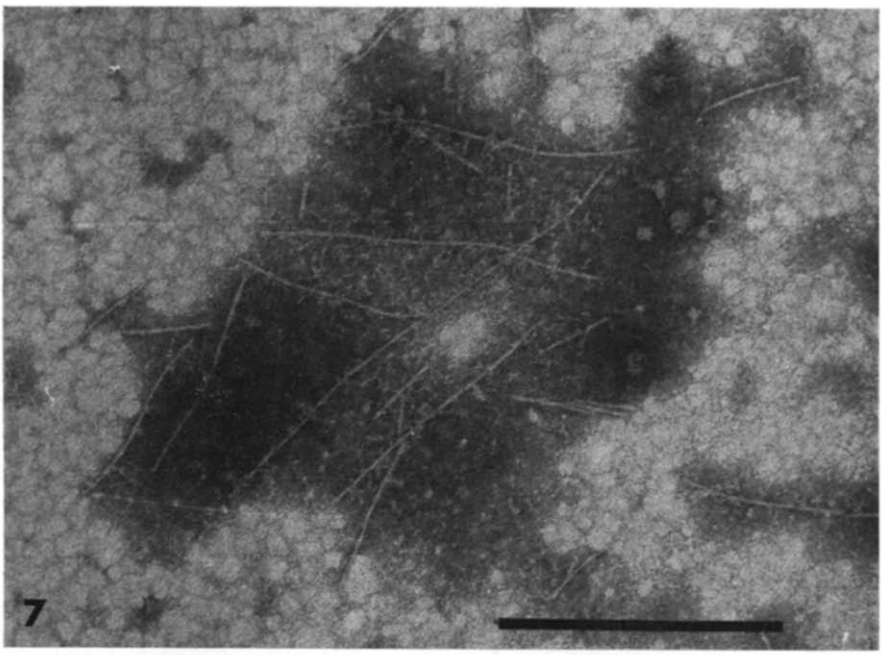


Fig. 5. The arrow head structure of myosin B in 5 mM KCl, 10 mM Tris–HCl (pH 7.4) and 1 mM $MgCl_2$. The bar in this and following figures indicates a measure of 0.5 μ.

Fig. 6. Disappearing arrow head structure. Myosin B was fixed 10 sec after the addition of 0.02 mM ATP to the suspension shown in Fig. 5.



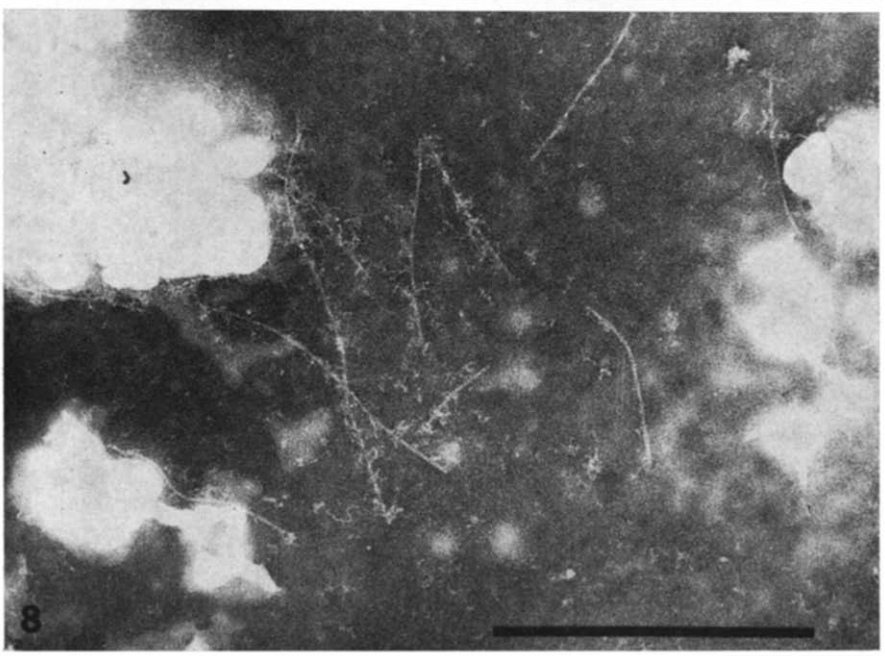


Fig. 7. Attachment of dense clusters to thin filaments. 1 min after the addition of ATP.

Fig. 8. Enhanced attachment of dense clusters to the thin filaments, suggesting some recovery of arrow head structure. 15 min after the addition of ATP.

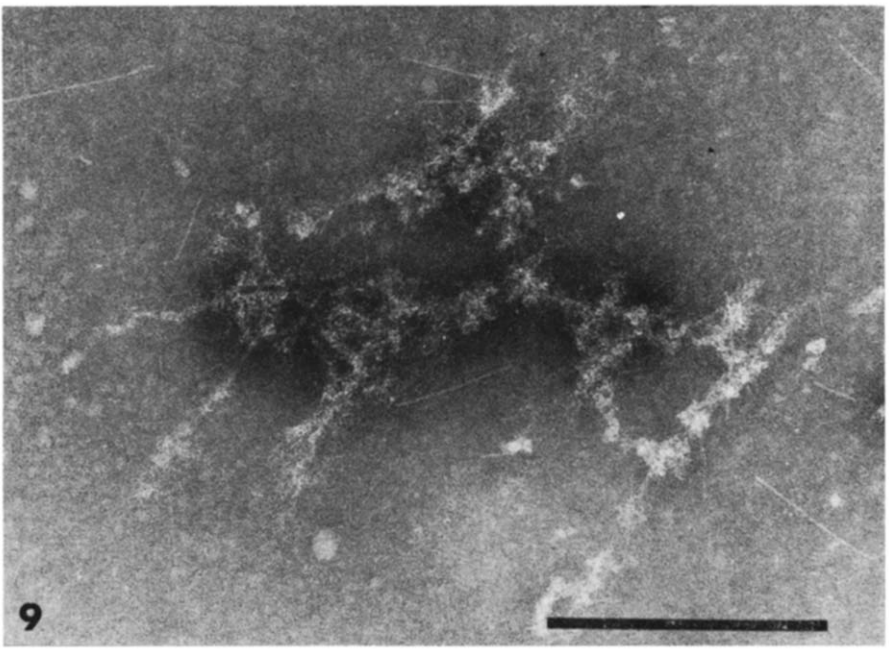


Fig. 9. Large aggregates formation. 30 min after the addition of ATP.

If the refractive index alters, the value of the polarization ratio is likely to vary. Therefore, the results in Fig. 4 can be interpreted as illustrating the increase in refractive index of the gel during the process of superprecipitation.

Although the electron microscope observations were reported by SPICER AND ROZSA[14], RICE *et al.*[4], HUXLEY[15], and IKEMOTO *et al.*[16], the experimental conditions used previously were different from those employed in this work. Furthermore, those observations reported do not agree with each other in all aspects. We, therefore, attempted an electron microscopic examination of myosin B which was treated under the conditions used for the determinations of turbidity and polarization ratio. When myosin B is suspended in a solution which contains 5 mM KCl, 1 mM $MgCl_2$ and 0.01 M Tris–HCl (pH 7.4) the appearance of myosin B is the "arrow head" type (Fig. 5) which was shown with the protein in 0.6 M KCl by HUXLEY[15]. Immediately after the addition of ATP, the "arrow head" structures tend to disintegrate (Fig. 6), then the electron dense clusters become attached to the thin filaments (Figs. 7 and 8). Subsequently, the dense clusters grow and some thin filaments are left free of the attachments (Fig. 9). According to the previous work[15,16], the thin filaments and the dense clusters are taken as representing actin filaments and myosin aggregates, respectively.

DISCUSSION

From the morphological observations described above, two distinct phases of superprecipitation can be distinguished: the first is represented by the tendency of arrow head structures to disintegrate; the second by the attachment and growth of

the dense clusters on the thin filaments. The first step appears to be consistent with the dissociation of actin and myosin which was observed in 0.1 M KCl and speculated to support the sliding theory of muscle contraction. It is a question, however, whether or not the dissociation of actin and myosin as observed with the isolated proteins, is, in fact, a step involved in the physiological muscle contraction. As pointed out by WEBER[17], the addition of ATP to actomyosin which is suspended in a medium containing no ATP does not necessarily initiate the reactions taking place in the actin–myosin system under the physiological condition where the concentration of ATP is kept virtually constant. It appears, therefore, unwarranted to speculate the direct relationship between the events which occur during superprecipitation and the reactions taking part in muscle contraction.

As described in the RESULTS, the change in turbidity does not detect the dissociation of myosin B on addition of ATP at 5 mM KCl, although when the KCl concentration is raised to 0.1 M, a decrease in turbidity at the outset of superprecipitation becomes apparent. The kinetic behaviour of turbidity change is first order in myosin B and follows the Michaelis formulae in terms of ATP, suggesting that the process reflected by the turbidity change does not involve interaction between the segments of myosin B, *i.e.*, the change occurs in a single unit mass. The angular dependence of the polarization ratio (Fig. 4) demonstrates that an increase in refractive index is a main factor contributing to the change in the optical characteristics during superprecipitation. It is noted, also, that the increase in the value of the polarization ratio is completed at the same time when the turbidity reaches its final value. Since a refractive index increase, in general, means an increase in the density of particles, the photometric experiments can be considered to detect a change in the density of myosin B, that is, a structural change in the protein. This structural change may occur at the stage where the "arrow head" structure disappears or, if the first step is the dissociation of actomyosin, this change may proceed during the step in which actin and myosin recombine, the latter step being rate limiting.

The growth of dense clusters obviously lasts much longer than the changes in turbidity and polarization ratio. In fact, if unpolarized light intensity is measured at angles between 25° and 45°, it increases continuously over a period of 1 h, whereas the the turbidity change ceases after less than 30 sec even at the lowest ATP concentration tested (0.005 mM). This indicates that the increase in the size of myosin B particles is independent of the turbidity change. It should be emphasized that the optical arrangement for the turbidity measurement is an important factor in deciding the type of physical parameter being detected[9].

Recently BRISKEY *et al.*[9] reported that superprecipitation proceeds in two steps: first the interaction of F-actin and myosin; and second, the structural change of the complex. For reasons stated above, the step involving the structural change is not the same as that of heavy aggregate formation. The morphological appearance of this heavy aggregate formation in which the dense clusters become larger, leaving some thin filaments free (Fig. 9) suggests a reversible association of the two components and strong attractive forces acting between the dense clusters. In this connection, the longer distance between the dense clusters attached to the thin filaments at the outset of the complex reformation (Figs. 6 and 7) could be an indication of structural specificity on the side of actin, although it may simply mean a strong intermyosin interaction and random association on the sites available.

The observations on the dilute actomyosin suspension as performed in this work have an obvious advantage over the previous experiments which used relatively high concentrations of protein, since dilution reduces the interactions between the actomyosin particles. For the reason stated earlier in this section, the results presented here cannot be directly related to the mechanism of muscle contraction. If a system of actomyosin dispersed in a solution could be obtained such that a phenomenon resembling the contraction–relaxation cycle were observed in the presence of constant amount of ATP, then the interaction of actin and myosin could be more readily discussed in relation to muscle contraction.

ACKNOWLEDGEMENTS

The authors wish to thank the Medical Research Council, the Muscular Dystrophy Association of Canada and the Life Insurance Medical Research Fund, for their support of this work.

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