FILAMENT FORMATION BY SLIME MOULD MYOSIN ISOLATED AT LOW IONIC STRENGTH

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SUMMARY

Myosin was isolated and purified from plasmodia of the slime mould Physarum polycephalum by a new method. This method is based on actomyosin extraction at low ionic strength after extensive washing, followed by the selective precipitation of myosin at pH 6.1 under relaxing conditions. The yield of myosin was 3–5 times higher than reported for other methods.

In contrast to earlier studies a remarkably strong tendency to filament formation was found for slime mould myosin, probably due to a better preservation of some structural properties during preparation. Conditions were worked out under which numerous filaments up to 4 fim in length can be produced. It was established that not only a gradual decrease of ionic strength may influence filament formation, but also pH, ATP concentration and the presence of divalent cations. Compared to the current filament models a difference exists in the structure of the filaments. No central bare zone can be found, and thus, they lack an apparent bipolarity. Along the entire filament there are lateral projections representing the head portion of myosin molecules. A clear periodicity with an axial repeat of about 14 nm was observed, indicating a highly ordered arrangement of these projections.

In this paper it is shown for the first time that myosin from one of the primitive motile systems is able to form aggregates of high structural order, indicating that the contraction of non-muscular actomyosin systems is not necessarily effected with oligomeric or randomly aggregated myosin. The possible role of myosin aggregation in vivo and the similarity of filament structure to that recently reported for myosin from vertebrate smooth muscle and striated muscle are discussed.

INTRODUCTION

Extensive morphological, biophysical and biochemical studies on the phenomena of cell motility have revealed that cytoplasmic streaming and amoeboid movement are based on the contraction of actomyosin-like protein complexes. One of the best known non-muscular motile systems is the slime mould plasmodium Physarum polycephalum which performs a very vigorous cytoplasmic streaming. The generation of motive force is closely connected with numerous fibrils and a filamentous network in the ectoplasmic region (for literature see Komnick, Stockem & Wohlfarth-Bottermann, 1973). This appears to be the morphological equivalent of the actomyosin system. The easy cultivation of slime mould plasmodia and the high amount of contractile protein which can be isolated make it highly suitable for investigations in this field.

could be precipitated by lowering the salt concentration (Hatano & Tazawa, 1968), the isolated myosin was found to be soluble in 0.03 M KCl and did not show any tendency to form aggregates larger than tri- or tetramers (Hatano & Takahashi, 1972). Similar properties were reported by Adelman & Taylor (1969a, b) for plasmodium myosin extracted at low ionic strength. Filamentous aggregates of slime mould myosin with a length of about 0.4-0.5 μm were first shown in actomyosin preparations which were kept in the relaxed state (Hinssen, 1970). These filaments were bone-shaped, with a central bare shaft and an assembly of myosin heads on either side. They demonstrated the general possibility of a tail-to-tail aggregation. These findings were confirmed with purified myosin (Nachmias, 1972). In addition, it was pointed out that divalent cations may influence filament formation, though variations of the Mg²⁺ and Ca²⁺ concentrations did not increase the length of aggregates. The previous results concerning the aggregation properties of slime mould myosin are still unsatisfying, because filaments appear to be necessary for efficient contraction of actomyosin (D’Haese & Komnick, 1972). Therefore, a new purification method for plasmodium myosin was developed and filament formation by this preparation was investigated.

MATERIALS AND METHODS

Plasmodia

Plasmodia of Physarum polycephalum were cultured by a combination of axenic submerged culture described by Daniel & Baldwin (1964) and the method of Camp (1936). Details are reported in a previous paper (Hinssen, 1972).

Protein concentration

Protein concentration was estimated according to Lowry, Rosebrough, Farr & Randall (1951) with serum albumin as standard.

Electrophoresis

SDS-gel electrophoresis was performed after the method described by Weber & Osborne (1969). The incubation was done in the presence of 8 M urea and 5 % β-mercaptoethanol for 30 min at 80 °C.

ATPase activity

Myosin ATPase was determined by measuring the liberated H⁺ by a pH-stat method (Eisenberg & Moos, 1967) using a Metrohm Combititrator 3D. The reaction was carried out in a solution containing 0.5 M KCl, 10 mM Tris-maleate buffer, pH 7.5, 2 mM CaCl₂, and 1 mM ATP at 25 °C.

Negative staining

Negative staining was done on carbon-coated Formvar films or carbon films with 1 % uranyl acetate after the method of Huxley (1963). For special purpose the grid was washed with a solution of 0.02 % cytochrome c in 0.1 % aqueous amyl alcohol (Moore, Huxley & DeRosier, 1970). Electron micrographs were taken on a Philips EM 300.
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Solutions

In the description of the myosin preparation the following solutions will be referred to:

(a) Washing solution: 0.035 M KCl, 1 mM cysteine, 0.035 M histidine buffer pH 7.0.
(b) Extraction medium: 0.1 M KCl, 1 mM cysteine, 5 mM ATP, 5 mM EGTA, 0.1 M histidine buffer pH 7.0.
(c) Dialysis solution: 0.035 M KCl, 0.5 mM ATP, 1 mM cysteine, 2 mM EGTA, 2 mM MgCl₂, 0.035 M histidine buffer pH 6.8.

RESULTS

Isolation and purification of myosin

For the isolation of myosin a method was used based on the extraction at low ionic strength described by Sobieszek (1972) for smooth muscle myosin. Plasmodia were homogenized with an equal volume of 'washing solution' containing in addition 1 mM CaCl₂. The homogenate was stirred for 15 min to allow the actomyosin to precipitate. Ten volumes of washing solution were added and after a few minutes of stirring the insoluble material was centrifuged down at 10,000 g for 40 min. The pellet was washed with 10 vol. of washing solution 3 times more to remove most of the soluble material. The final pellet was suspended in 2 or 3 vol. of 'extraction medium' and homogenized thoroughly. After 3 h of extraction the suspension was centrifuged at 30,000 g for 2 h. The clear supernatant of this centrifugation will be referred to as 'crude extract'. The crude extract was dialysed against 10–15 vol. of 'dialysis solution' overnight, which led to a slight turbidity. The pH of the sample was then slowly adjusted to 6.1. After stirring a precipitate formed which was centrifuged down at 7000 g for 30 min. The pellet was washed once with dialysis solution pH 6.1 and subsequently extracted again for about 20 min at pH 7.0 with extraction medium containing 0.2 M KCl. After centrifugation a clear and colourless solution of almost pure myosin was obtained. This fraction was used for nearly all experiments. Further purification is possible by repeating the last extraction step.

The isolation procedure was controlled by SDS-gel electrophoresis. Fig. 2A shows that in the starting material there is a remarkably strong band with an electrophoretical mobility corresponding to that of isolated slime mould actin. In addition to many other unidentified proteins there is a band with a molecular weight of about 220,000 which probably represents the heavy chains of slime mould myosin (Nachmias, 1972). The gel of the crude extract (Fig. 2B) indicates that there is a relative enrichment of actin and myosin, though a great deal of actin is already lost during the preparation procedure. Another strong band appears below the actin with a molecular weight of about 35,000, which is in the range of muscle tropomyosin (Weber & Osborne, 1969). Almost complete separation of the actin from myosin and further purification takes place even after one pH-precipitation and with the following extraction (Fig. 2C).

Though the crude extract contained a rather high amount of actin as shown by electrophoresis, in electron-microscopic controls only a few, and very short, actin filaments could be detected. Consequently, a separation of actin and myosin could not be attained by centrifuging down actin at high speed as has been described in other
methods (A. Weber, 1956; Sobieszek, 1972). Therefore the spontaneous aggregation of the myosin into filaments and amorphous precipitates which could be observed in this extract after standing for 2 days in the cold room was used for purification. The aggregation was supported and accelerated by the dialysis step described above. The filaments form larger precipitates if the pH is lowered to 6.1 and can thereby be separated by centrifugation. The essential point was to keep permanent relaxing conditions without addition of too much ATP because a higher ATP concentration prevented filament formation. The presence of EGTA in all relaxing solutions was necessary, because – in contrast to muscle myosin – it strongly inhibits the slime mould myosin ATPase (Hatano & Ohnuma, 1970; Adelman & Taylor, 1969b). The yield of myosin was about 25 mg per 100 g of fresh plasmodia. This is 3–5 times higher than reported earlier (Hatano & Ohnuma, 1970; Adelman & Taylor, 1969b). The ATPase activity was determined to be 0.7 µmol Pi/min/mg protein under the conditions described above. This value is in the range of previous measurements for Physarum myosin (Hatano & Ohnuma, 1970; Adelman & Taylor, 1969b). The myosin isolated by this procedure exhibits characteristics generally reported for myosin. It combines with muscle F-actin forming the typical arrowhead structure. Thread models (according to Weber, 1935) made of this synthetic, hybridized actomyosin show rapid contraction after the addition of ATP (Hinssen & D’Haese, unpublished results).

Conditions for filament formation

As mentioned before, the myosin isolated according to the former methods has only a very weak tendency to form filaments. On the other hand even in the crude extract of our preparation numerous myosin filaments are present which are larger than all aggregates described previously. These filaments are very sensitive to changes in ionic strength, ATP concentration and variations of pH. Therefore, it was necessary to determine the most suitable conditions for filament formation. Increase of the KCl concentration up to 0.15 M leads to total dissolution of the filaments. A similar effect was observed when the ATP concentration was raised above 3 mM. At pH values other than 7.0 the filaments become unstable also: disintegration of the structure is observed at pH 7.6, whereas precipitates are formed below pH 6.5. Though divalent cations were not absolutely necessary, it was found that filament formation is improved by Mg^{2+} concentrations up to 2 mM and by Ca^{2+} up to 0.5 mM. However, after some time, Ca^{2+} leads to a partial precipitation of the filaments. Since it was not the intention in this study to examine the effects of various factors in detail we adopted for the following experiments one combination which was found to be most suitable for filament formation. We used a KCl gradient ranging from 0.2–0.05 M in the presence of 0.5 mM ATP, 2 mM MgCl₂, and 35 mM histidine buffer at pH 6.9. In certain cases 0.5 mM CaCl₂ was also added. ATP gradients were less effective. The same holds true for the gradual decrease of pH at constant ionic strength. In conclusion, there is a rather strictly defined range of conditions under which myosin filaments can be produced and under which they are stable.
Filaments from purified myosin and their fine structure

To make filaments as long as possible it was necessary to decrease the KCl concentration very slowly (within 1–2 days). This was performed by dialysing the sample against the above mentioned solution with 0.2 M KCl, and subsequent dropwise addition of the same solution without KCl. The protein concentrations of the samples were normally about 1.5–2.0 mg/ml.

A typical result of such an experiment is shown in Fig. 4. Numerous spindle-shaped filaments are found, and there is almost no precipitation of the filaments into clusters. There is a remarkable variation of filament length and, therefore, the relative frequency of the filaments was plotted against their length. Fig. 1 shows that the lengths of the filaments range from 0.3 to 2.0 μm with a clear maximum at 0.65 μm. The asymmetrical shape of the distribution curve with the flat slope of the descending part indicates that more than half of the filaments (70%) are longer than the value of the curve maximum; 32% of the filaments exceed one micron. The absolute length of the filaments may vary somewhat from experiment to experiment, depending on the rate of lowering the ionic strength. Extension of this time and especially the presence of Ca²⁺ leads to much longer filaments. In these experiments filaments of 3–3.5 μm are often found, the largest ones measuring around 4 μm (Figs. 14–20). Unfortunately, the number of precipitates of myosin increases with the average length, so that a statistical evaluation could not be made from these samples.

At higher magnifications it can be observed that the filaments are not clearly
bipolar and lack a central bare zone (Figs. 5, 6, 14–20). Along their entire length they are covered with lateral projections, which extend about 10–15 nm from the filament body – depending on the staining conditions – and which may represent the head part of myosin molecules. The diameter of the filaments measured without the lateral projections ranges up to 30 nm. At lengths above 1 μm this diameter remains rather constant. Beside the filaments there is a molecular background which, because of the relative purity of the fraction, most likely represents some non-aggregated myosin.

If cytochrome c is used during negative-staining preparation (see Materials and Methods) a regular periodic banding along the whole filament can be observed (Figs. 9, 10, 14–20). The repeat of the periodicity was 14–15 nm. This may be caused by a regular arrangement of the myosin heads. For demonstrating the periodicity the Ca²⁺-treated samples are much more favourable, though the banding is also present in some of the filaments obtained without Ca²⁺.

Short myosin filaments were obtained within 1 h when the ionic strength of the sample was decreased rapidly by dialysing immediately against a solution with 0.035 M KCl, 0.035 M histidine buffer pH 6.5, 2 mM MgCl₂, 1 mM CaCl₂ and 0.5 mM ATP (Fig. 3). However, under these conditions, the extension of dialysis for more than 1 h caused precipitation of myosin.

The filaments produced by rapid dialysis against a steep gradient resembled in structure those obtained from prolonged dialysis against a shallow gradient. Their lengths ranged from 0.5 to 1.0 μm (Figs. 9, 10). Some of the shorter ones displayed a clearly asymmetrical shape in that on opposite sides they had one smooth edge free of projections at either end of the filament (Figs. 7, 9). These smooth edges could be found also on the larger filaments (Figs. 18, 20), indicating that the short asymmetrical filaments may be early stages of development. This has already been described by Sobieszek (1972) for the filaments of smooth muscle myosin preparations. All filaments of plasmodium myosin described in earlier studies (Hinssen, 1970; Nachmias, 1972) which were found in actomyosin preparations or isolated myosin, were of the bone-shaped type and measured about 0.4–0.5 μm in length. The degree of structural order was very low in these filaments and their bipolarity seemed to be caused by parallel alignment of the myosin rods, which led to a more or less random distribution of myosin heads at both ends of the filaments. This type of filament was very rarely found in our preparations (Fig. 13) but their number increased when very rapid lowering of the ionic strength was performed, i.e. by dilution of the sample. Large numbers of bone-shaped filaments could be formed from myosin which had been isoelectrically precipitated or after short trypic digestion of the myosin (Figs. 11, 12). In these cases even a very slow decrease of ionic strength did not lead to longer filaments.

**DISCUSSION**

In this paper a method is described for the isolation of myosin from the slime mould *Physarum polycephalum*. This method is based on the solubility of actomyosin at low ionic strength in the presence of ATP, whereas during the preceding extensive
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washing procedure it remains insoluble. The separation of actin and myosin is obtained by precipitation of the myosin at lowered pH under relaxing conditions.

Isolation of plasmodium myosin was attained earlier by extracting actomyosin at high ionic strength and separating the myosin by the method of A. Weber (1956) (Hatano & Ohnuma, 1970). Purification of myosin was carried out by isoelectric precipitation. An alternative method exists already (Adelman & Taylor, 1969a, b), which involves direct extraction of actomyosin with 50 mM pyrophosphate and fractionation of myosin by column chromatography.

The general advantages of the method presented here are a rather simple and short preparation, an easy and nearly complete separation from the actin and a comparable high yield. In addition, the tendency to form filaments was found to be remarkably higher than in the case of myosin prepared by one of the other methods. In these cases the loss of this property may be due to worse preservation, because either isoelectric precipitation or the use of pyrophosphate can induce irreversible changes in the myosin (A. Sobieszek, personal communication; Dörö & Portzehl, 1954).

On the other hand, the good preservation of myosin, indicated by the formation of arrowhead complexes in combination with actin, and the contraction of thread models, suggests that the capacity for filament formation is not an artificial property caused by our preparative method.

The knowledge of myosin aggregation is an essential prerequisite for the explanation of the working mechanisms in actomyosin systems. Especially in those systems which are of lower structural order than cross-striated muscle, the state of myosin is not sufficiently investigated. Though, for instance, vertebrate smooth muscle myosin is able to form thick filaments in vitro (Kaminer, 1969; Sobieszek, 1972; Sobieszek & Small, 1973a, b) there is much controversy about its state in situ, and uncertainty whether it forms only small aggregates, ribbon-like structures or thick filaments (Panner & Honig, 1967, 1970; Cooke, Chase & Cortés, 1970; Cooke & Fay, 1972; Devine & Somlyo, 1971; Rice et al. 1970; Lowy & Small, 1970; Small & Squire, 1972).

Concerning the slime mould material, various attempts were made to obtain myosin filaments comparable to muscle. The first step towards this goal was the demonstration of short bipolar filamentous aggregates in actomyosin preparations (Hinssen, 1970) and in purified myosin fractions (Nachmias, 1972). By this means slime mould myosin was shown to be able to form tail-to-tail aggregates similar to synthetic filaments from muscle myosin.

In such preparations the proportion of aggregated myosin was very small and the filaments – consisting of a few molecules only – were of low structural order. Recent electron-microscopic observations also indicate the in situ existence of myosin aggregates in both glycerinated and aldehyde-fixed plasmodia (Alléra & Wohlforth-Bottermann, 1972; Kessler, 1972). In the low ionic strength extracts an almost complete aggregation of myosin into highly ordered filaments up to 4 µm in length can be obtained. The conditions of filament formation are somewhat critical compared to muscle. They correspond to the specific solubility properties of slime mould myosin, which partly resemble those of vertebrate smooth muscle (Barany, Barany, Gaetjens
& Bailin, 1966). The dissolving experiments show that in addition to the ionic strength there are a number of factors to be kept in mind. Though the reported conditions are not necessarily optimal for filament formation, it should be pointed out that the ionic concentrations in which the filaments are stable are similar to those found in the living plasmodia (Hatano & Totsuka, 1972).

ATP in concentrations above 3 mM begins to dissolve the myosin filaments at low ionic strength, and vice versa a gradual decrease of the ATP concentration can be used to obtain filament formation. It is most likely that the filaments, which were observed in actomyosin preparations kept in relaxing conditions for some time (Hinssen, 1970), were produced by such a gradient caused by the splitting of ATP and not by the rapid lowering of ionic strength.

In this paper 2 types of morphologically different filaments have been reported from slime mould myosin which can be produced under various conditions: (1) The bone-shaped filaments which are identical to those reported in previous studies. They have a rather constant length and a bare zone of about 0.2 μm. The random distribution of myosin heads on either end of the filaments causes a clear bipolarity. (2) The spindle-shaped filaments with the following main features. (a) A variable filament length which depends on the conditions used for filament formation. The length is theoretically unlimited. (b) An apparent lack of morphological bipolarity caused by the distribution of myosin heads along the entire filament so that no bare zone can be observed. (c) A regular arrangement of myosin heads which leads to a strong periodicity with a repeat of about 14 nm, indicating a high degree of structural order.

The structure of the spindle-shaped filaments resembles closely that found by Sobieszek (1972) for smooth muscle myosin isolated by a similar method. Concerning the lack of a central bare zone it differs from the natural and synthetic filaments from cross-striated muscle (Huxley, 1963; Kaminer & Bell, 1966), though recent preliminary results indicate that purified cross-striated muscle myosin – containing no C-protein – is able to aggregate in the same manner (Moos, 1973). In addition, X-ray diffraction studies reveal a clear 14.4-nm periodicity in cross-striated (Huxley & Brown, 1967) and vertebrate smooth muscle (Lowy, Poulson & Vibert, 1970) which arises from the presence of ordered myosin aggregates, showing that at least this property of the synthetic filaments is involved in the organization of myosin in living muscle. This shows that the filament structure of slime mould myosin is not an artifact, but may represent the natural aggregation of pure myosin.

The molecular architecture of the filaments and the relations between the different filament types are still unclear and an explanation should be left to further, more detailed investigations.

The results reported here show that contraction of non-muscular actomyosin systems is not necessarily effected with oligomeric or randomly aggregated myosin. In principle, slime mould myosin has the same aggregation properties as myosin from muscle and it seems unlikely that plasmodia should make no use of them. Because of the ephemeral nature of the contractile structures in slime mould, which build up and disappear in close connexion to the protoplasmatic shuttle streaming, the
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existence of very long filaments in the living system is not assumed, but the mode of aggregation could be the same as in the in vitro experiments.

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REFERENCES


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Figs. 2 and 3. For legend see p. 124.
Fig. 2. SDS-gel electrophoresis on 7-5% gels showing the purification of slime mould myosin. A, whole slime mould. The strong band in the middle of the gel has a molecular weight of about 45,000 and was identified as actin by co-electrophoresis with purified slime mould actin. B, crude actomyosin extract. A relative enrichment of myosin has already taken place. C, myosin after one purification step. Almost complete separation from actin and other substances is obtained.

Fig. 3. Survey micrograph of short myosin filaments produced by direct dialysis against a medium of low ionic strength. ×10,000.

Fig. 4. Survey micrograph of myosin filaments obtained by prolonged decrease of ionic strength. No Ca^{2+} was present during filament formation. ×10,000.
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In Figs. 5–20, the scale lines are equivalent to 0.2 μm.

Figs. 5, 6. Higher magnifications of filaments as shown in Fig. 4. Lateral projections can be seen along the whole filament, there is no central bare zone. × 85,000.

Fig. 7. Short asymmetrical myosin filament. Note the smooth edge (indicated by arrows) on either end of the filament, whereas lateral projections are present in the middle of the filament. × 100,000.

Fig. 8. Small myosin aggregate consisting of a few molecules only. Compare with Figs. 11–13. × 125,000.

Figs. 9, 10. Small myosin filaments from a sample containing Ca²⁺ during filament formation. A periodicity can be observed along the filaments. Note the asymmetrical shape of the filament in Fig. 9 and the smooth edges between the arrows. × 100,000.

Figs. 11–13. Short bipolar myosin filaments obtained with isoelectrically precipitated myosin (Fig. 11), after very short trypic digestion of the myosin (Fig. 12) or found very seldom in normal samples after rapid decrease of ionic strength (Fig. 13). There is no structural difference between the 3 filaments. Comparison with Fig. 8, showing a filament of about the same size, indicates a general difference in the mode of aggregation. × 125,000.
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Figs. 14–20. Long myosin filaments obtained in the presence of Ca\(^{2+}\). The periodical arrangement of the cross bridges can be seen on the whole filaments (arrows indicate smooth edges). Figs. 14–16, \(\times 60000\); Figs. 17–20, \(\times 80000\).