The actin-activated ATPase of co-polymer filaments of myosin and myosin-rod

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The actin activated ATPase of myosin at low ionic strength shows a complex dependence on actin concentration, in contrast with the simple hyperbolic actin activation kinetics of heavy meromyosin and subfragment-1. To investigate how the aggregation of myosin influences the actomyosin ATPase kinetics, we have studied the actin-activated ATPase of mixed filaments in which the myosin molecules are separated from each other by co-polymerization with myosin rod. Electron microscopy of co-polymer filaments, alone and bound to actin, indicates that the myosin heads are distributed randomly along the co-polymer filaments. The actin-activated ATPase of myosin decreases

with increasing rod, approaching a plateau of about 30 % of the control at a rod/myosin molar ratio of 4:1. The decrease in ATPase persists even at V_{max} , the extrapolated limit at infinite actin, indicating that it is not due merely to the loss of co-operative actin binding. Furthermore, the actin dependence of the ATPase still shows a biphasic character like that of control myosin, even at a rod/myosin ratio of 12:1, so this complexity is not probably due solely to the structural proximity of myosin molecules, but may involve a non-equivalence of myosin heads or myosin molecules in the filament environment.

INTRODUCTION

Most studies of the enzymology of myosin-actin interaction have been done using soluble fragments of myosin, HMM (heavy meromyosin) and S-1 (subfragment-1), which are more amenable to kinetic analysis than is intact myosin. An unanswered question with this approach has always been the relationship between the ATPase kinetics of these fragments and intact myosin. In particular, there are qualitative features of the actin-activated myosin ATPase which are not observed with HMM or S-1. Among these are a non-linear time course of actin-activated ATPase at low ATP concentrations (Kinoshita et al., 1969; Strzelecka-Golaszewska et al., 1979), and a complicated biphasic, or even polyphasic, actin dependence of the myosin ATPase (Pope et al., 1981; Cheung and Reisler, 1983; Strzelecka-Golaszewska and Piwowar, 1984).

These kinetic differences between myosin and its soluble fragments could, of course, be due to proteolytic damage during preparation of HMM and S-1, but they might also be related to the fact that myosin, at low ionic strength, is assembled into filaments. Several laboratories have suggested ways in which filament formation might influence the actin-activated ATPase. The complicated actin-dependence of myosin ATPase might arise merely from the presence of multiple sites of interaction between myosin and actin filaments, because attachment of actin at one site could cause other nearby myosin heads to 'see' a higher effective actin concentration than is present in the bulk solution (Sleep et al., 1980; Cheung and Reisler, 1983; Root et al., 1991). Alternatively, a more subtle co-operative interaction between myosin molecules within the thick filament might also occur, as pointed out by Stępkowski et al. (1984) and Root et al. (1991). Finally, even though two-headed HMM generally shows simple kinetics (Moos, 1972), the filament structure might

impose an asymmetry between the two heads of myosin and thereby cause complex kinetics (Schaub and Watterson, 1981).

To test some of these possibilities we have used co-polymer filaments in which the myosin is 'diluted' by myosin rods, which do not interact with actin, thus separating the myosin molecules from each other. The observations that myosin rods can self-assemble in a similar manner to intact myosin (Ward and Bennett, 1989) and that rod molecules can be exchanged between filaments of rod and myosin at low ionic strength (Patil et al., 1987) support the assumption that co-polymer filaments can serve as a reasonable representation of thick filaments with a reduced number of myosin heads. In these co-polymer filaments, the probability that an actin filament could simultaneously interact with many myosin molecules in the same filament is reduced, and interactions between myosin heads within the filament should not occur. However, any effects of the rod part of the myosin molecules on the kinetics of adjacent heads would remain. We have examined the actin-activated ATPase kinetics of myosin-rod co-polymer filaments as a function of the proportion of added rod. In addition, the copolymers were examined in the electron microscope, both alone and mixed with actin, to monitor their general appearance and the distribution of myosin molecules along them.

MATERIALS AND METHODS

Preparation of proteins

Skeletal-muscle myosin was prepared from rabbit back and leg white muscles and purified by DEAE-Sephadex chromatography (Offer et al., 1973), and myosin rod was obtained by papain digestion of this myosin followed by alcohol precipitation (Margossian and Lowey, 1982). Actin was purified as described by Pardee and Spudich (1982).

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Figure 1 Electron micrographs of rod, myosin and co-polymer filaments

Filaments formed from: rod alone (a), 1:1 rod/myosin mixture (b); myosin alone (c); 4:1 rod/myosin mixture (d); 12:1 rod/myosin mixture (e). The scale bar represents 66.7 nm.

Figure 2 Electron micrographs of co-polymer filaments bound to actin

Rod-myosin copolymer filaments were mixed with actin (0.5 mol/mol of myosin), in the absence of ATP, before application to electron-microscope grids. Panels show: 1:1 rod/myosin (a and b); 4:1 rod/myosin (c and e); 12:1 rod/myosin (d and f). The scale bar represents 66.7 nm.

Filament formation

Myosin or myosin/rod mixtures were prepared in 0.5 M KCl/20 mM Mops (pH 7.0)/1 mM MgCl_2 at a myosin concentration of 5 mg/ml, and filaments were formed by diluting this solution to 0.1 M KCl and 1 mg/ml myosin at constant [Mops] and $[\text{MgCl}_2]$.

Electron microscopy

For electron microscopy, the filament suspensions were diluted to the concentration 0.2 mg/ml of protein, applied to grids coated with formvar and carbon, and negatively stained with 1% (w/v) uranyl acetate. A JEOL JEM 1200 EX electron microscope was used at an accelerating voltage of 80 kV, and pictures were taken at a magnification of $30000\times$.

ATPase measurements

The ATPase measurements were performed with the use of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ essentially by the method of Chock and Eisenberg (1979). The ATPase reaction mixtures contained 0.2 mg/ml myosin, 2 mM MgATP, 0.1 M KCl, 1 mM MgCl_2 and 20 mM Mops, pH 7.0, in a total volume of 0.5 ml, and were continuously stirred in a bath at 25 °C. The reactions were started by addition of the filament suspension, and 0.1 ml samples were taken at appropriate times and transferred to tubes containing the phosphate assay mixture, including the organic layer. Each tube was immediately vortex-mixed for 30 s, 0.1 ml of the organic layer was transferred to a scintillation vial, and 3 ml of scintillation cocktail were added for the radioactivity measurement. The reaction rate was calculated from the slope of a linear regression of four time points. The time course of the ATPase was nearly linear under our conditions, over the period of the measurements, even at the highest actin concentration. The correlation coefficients of these regressions were never less than 0.98. No more than 10% of the ATP was hydrolysed during the assay, even at the last time point of the fastest reactions.

RESULTS

The structure of co-polymer filaments: electron microscopy

As a basis for interpreting the effects of added rod on the myosin ATPase activity, we have examined the structure of the mixed filaments in the electron microscope. In particular, we tried to ascertain whether rod and myosin actually do co-polymerize and, if so, whether the myosin is distributed randomly along the filaments or is localized in clusters. In Figure 1, filaments of myosin alone, rod alone and myosin/rod mixtures are shown. The control myosin filaments (Figure 1c) show the familiar bipolar structure with a bare zone, whereas rod filaments (Figure 1a) appear smoother, longer and thicker. The surface of the copolymeric filaments (Figures 1b, d and e) is rougher than the control rod filaments, and in some cases projections similar to those observed on control myosin filaments are visible (see the arrows in Figure 1).

These micrographs show that myosin and rod do indeed co-polymerize, but the distribution of myosin heads on the mixed filaments could not be clearly seen. In Figure 2, actin was added at a 0.5:1 molar ratio to myosin to help reveal the distribution of myosin heads. The 1:1 rod/myosin filaments formed bundles with actin, showing well defined 'arrowhead' structures (marked by arrows), although the arrowheads are seen to be disrupted in

some places (Figures 2a and 2b). At 4:1 rod/myosin, arrowheads are only rarely seen (Figures 2c and 2e), and none were observed with 12:1 rod/myosin filaments. In both 4:1 and 12:1 rod/myosin filaments, actin binding was evident at multiple sites distributed irregularly along the filaments and, in some cases, apparent sites of binding of individual myosin heads to actin could be identified (e.g. double arrows in Figure 2d).

These observations support the view that the myosin in the mixed rod/myosin filaments is indeed distributed relatively randomly along the filaments rather than in localized regions.

ATPase activity of co-polymer filaments

Figure 3 shows the change in the actin-activated MgATPase activity of copolymer filaments with increasing rod/myosin ratios at constant myosin concentration. As stated in the Materials and methods section, every point in Figure 3, and in Figure 4 as well, is obtained from a linear regression of the initial time course of the reaction, with a regression coefficient of at least 0.98. At the ionic strength we used [0.1 I (100 mmol/litre) KCl], the reaction

Figure 3 Dependence of actin-activated ATPase activity of myosin on the molar ratio of rod to myosin in co-polymer filaments

The actin concentration was 10 μM and that of myosin was 0.42 μM . The ATPase activity was measured in medium containing 0.1 M KCl, 20 mM Mops, pH 7.0, 1 mM MgCl_2 and 2 mM MgATP.

Figure 4 Actin-dependence of myosin ATPase in co-polymer filaments

The Figure shows a double-reciprocal plot of the actin dependence of myosin ATPase for filaments formed from myosin (○), a mixture with a myosin/rod molar ratio of 1:6 (Δ); and a mixture with a myosin/rod molar ratio of 1:12 (□). The reaction was allowed to proceed as described in the Materials and methods section. (b) Contains expanded high-actin-concentration phase of the curves. Symbols in both (a) and (b) are the same.

Table 1 Kinetic parameters ($V_{\max}$ and K_{app}) derived from the high actin concentration range presented on Figure 4

The non-linear regression was performed using the Michaelis-Menten kinetics option in the ENZFITTER program. Values for kinetic constants are means $\pm$ S.E.M. values.

Filament preparation	$V_{\max}$ (s^{-1})	K_{app} (μM)	Number of points used for non-linear regression
Myosin	6.3 ± 0.9	196 ± 48	9
Myosin/rod ratio 1:6	3.8 ± 0.3	278 ± 31	9
Myosin/rod ratio 1:12	2.6 ± 0.2	209 ± 23	14

in this period shows linearity, even at the highest actin concentrations. Addition of rod significantly decreases the ATPase activity of myosin at a rod/myosin molar ratio of 1:1, and above 4:1 the ATPase reaches a plateau of about 30% of the control ATPase activity in the absence of rod.

The actin-dependence of myosin ATPase is shown on Figure 4 for control myosin filaments and co-polymeric filaments containing 6 or 12 mol of rod/mol of myosin. Although the presence of rod reduces the ATPase, the shape of the double-reciprocal plot is not changed; the non-linearity is not removed, even at a rod/myosin molar ratio of 12:1. Increasing the rod/myosin ratio from 6:1 to 12:1 causes little further change in the actin-dependence of the ATPase activity suggesting that, at 6:1, myosin molecules in these filaments are sufficiently separated so that they bind actin independently of each other. The inhibitory effect of adding rod to myosin filaments was also observed by Root et al. (1991).

Since the reciprocal plot is non-linear, it is difficult to derive meaningful single values of K_{app} or $V_{\max}$ from these data. However, to obtain an estimate of the maximum ATPase at actin saturation, a non-linear regression was applied to the data for the higher actin concentrations, from 10 to 200 μM . The resulting values of $V_{\max}$ are presented in Table 1. Values of K_{app} are also shown, although their physical significance in this complex system is much less clear. As can be seen, $V_{\max}$ was considerably lower for co-polymer filaments than for control myosin filaments. This finding contrasts with the report of Root et al. (1991) that $V_{\max}$ is unchanged by admixture of rod to myosin filaments, but their measurements were made at lower actin concentrations (only up to 70 μM instead of 200 μM) and under different conditions of pH and ionic strength, and their ATPase rates were not corrected for the ATPase in the absence of actin (Moos, 1972).

DISCUSSION

The experiments presented here were planned on the assumption that rod and myosin would co-polymerize randomly so that myosin molecules would be separated from each other within the mixed filaments. It is difficult to verify this assumption definitively, but it is supported by the published reports that rod filaments are built of subfilaments similar to those in myosin filaments (Ward and Bennett, 1989), and that when preformed myosin filaments and rod filaments are mixed at low ionic strength, rod molecules can be exchanged into the myosin filaments (Patil et al., 1987).

The electron micrographs shown here also suggest that myosin is distributed along the length of the co-polymer filaments, not clustered in localized regions. Although it is difficult to resolve individual myosin heads on the surface of the mixed filaments,

added actin filaments appeared to bind to the co-polymer filaments at widely separated sites distributed along their lengths. While actin bound to filaments of myosin alone was often seen to be decorated in an 'arrowhead' pattern, very few such arrowheads could be seen with mixed filaments containing 6:1 rod/myosin, and none were seen at a rod/myosin ratio of 12:1. Since formation of arrowheads requires groups of seven adjacent myosin heads (three to four molecules) (Moore et al., 1970), the absence of arrowheads implies that clusters of more than three myosin molecules probably do not exist in the mixed filaments, although we cannot exclude the presence of groups of two or three adjacent myosin molecules.

In considering the implications of the ATPase data, we assumed that myosin molecules are indeed randomly diluted by rod in the mixed filaments. The first question is, why do myosin molecules in a myosin filament have a higher actin-activated ATPase than individual myosins incorporated into filaments of rod? The simplest answer is that the presence of adjacent myosins allows co-operative binding of actin, i.e., that tethering of an actin filament to one myosin molecule increases the effective actin concentration available to adjacent myosin molecules (Sleep et al., 1980; Cheung and Reisler, 1983). This idea leads to the prediction that increasing the actin concentration should overcome the inhibition by rod because, at infinite actin, even single myosin molecules should be fully activated. In other words, addition of rod should not change $V_{\max}$, and Root et al. (1991) reported that this was indeed the case. However, in the present study, in which we have extended the measurements to higher actin concentration (200 μM as compared with 70 μM), we found that $V_{\max}$ is actually significantly reduced by the addition of rod. [The discrepancy may also depend on the difference in ionic strength and pH and the fact that Root et al. (1991) did not correct their ATPase rates for the activity in the absence of actin (Moos, 1972)]. Therefore, we do not believe that co-operative binding of actin can account for the higher ATPase of myosin filaments than myosin-rod co-polymers.

As another simple explanation for the ATPase inhibition by rod, one might imagine that the myosin in the co-polymer filaments can be 'buried' by overlying rod so that it is completely inaccessible to actin. However, this explanation, too, is unlikely. While it is indeed consistent with the reduced $V_{\max}$, it would predict that, if enough rod is added, all the myosin could eventually be inactivated, whereas, in fact, the ATPase reaches a plateau at about 4:1 rod/myosin, beyond which further addition of rod causes little further inhibition.

A more likely interpretation is the adjacent myosin molecules within the filament interact directly. Root et al. (1991), in their study of the kinetics of mixed minifilaments containing normal and SH-1 modified myosin, pointed out that interactions between adjacent myosins might occur through conformational changes within either the myosin or actin filament. In addition, the binding and cycling of one myosin head on actin might exert a force on adjacent myosin-actin cross-bridges which could influence their ATPase activity (Eisenberg and Hill, 1978). At this point, unfortunately, there is not sufficient evidence on which to base a more definite answer as to why the $V_{\max}$ of myosin molecules is increased by their close packing in a myosin filament.

Our second major finding is that rod-myosin co-polymer filaments in which myosin-myosin interactions have probably been largely eliminated, as indicated by the levelling off of the ATPase activity with increasing rod content (Figure 3) and the disappearance of arrowhead structures in the electron micrograph (Figure 2), shown the same sort of complex actin-activation kinetics as are seen with myosin. Hence this kinetic behaviour does not seem to depend on either multi-site binding

of actin to myosin filaments or myosin–myosin interactions. Even when the myosin heads are separated by intervening rod, they do not seem to behave identically and independently with respect to actin binding. One can speculate that the assembly of rods or myosin tails in the core of the filament may be such that the myosin molecules are not all in the same environment, or, maybe, the two heads of a myosin molecule are made non-equivalent by the incorporation of the molecule into a filament (Schaub and Watterson, 1981). On the other hand, we must again acknowledge that we have not excluded the possibility that myosins could occur in pairs or trimers, even when co-polymerized with rod, in which case a direct interaction between myosin molecules might still be responsible for the co-operative kinetics; however, the exchange of rod molecules between filaments of rod and myosin (Patil et al., 1987) argues against this possibility.

In summary, a myosin filament is apparently not equivalent simply to a set of HMMs attached to a passive structural support. The differences between the kinetics of myosin and its soluble fragments, HMM and S-1, cannot be explained simply by the multi-site binding of myosin filaments to actin. Rather, the actin-activated ATPase of individual myosin molecules seems to be modified in a complex manner by their assembly into a filament.

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