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Myosin–paramyosin cofilaments: Enzymatic interactions with F-actin

(enzymology of filaments/heavy meromyosin subfragment 1 probe)

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ABSTRACT **The interaction between paramyosin and myosin has been studied by enzymological methods. Clam adductor paramyosin inhibits the actin-activated, Mg^{2+}-requiring ATPase of both clam adductor and rabbit skeletal muscle myosins. Myosin and paramyosin must be rapidly coprecipitated for this inhibition. Incubation with F-actin in the absence of ATP does not alter this effect. This inhibition follows a hyperbolic function with respect to paramyosin concentration. Slow precipitation by dialysis of myosin and paramyosin together leads to copolymers with actin-activated ATPase equivalent to that of slowly formed myosin filaments. Both kinds of slowly formed filaments have enzymatic properties distinct from those of the rapidly precipitated proteins.**

Paramyosin is competitive with F-actin for their effects upon myosin. The apparent affinity of myosin for F-actin is markedly reduced by association with paramyosin, but the extrapolated maximal velocity of actomyosin is unaffected. The specificity of this inhibition is strongly suggested by marked quantitative differences between native and cleaved paramyosins. No inhibition of intrinsic myosin ATPase by paramyosin is seen.

These studies suggest that at least two types of condition-dependent association between myosin and paramyosin are possible. One class of interactions is associated with enzymic inhibition in rapidly coprecipitated filaments, whereas slowly formed cofilaments exhibit catalytic activity similar to that of identically treated myosin and have a characteristic 14.5 nm axial repeat.

Paramyosin is a major structural component of muscles from a diverse range of invertebrate species including nematodes (1) and insects (2), yet the extent of its functions during muscle activity is not known. We describe here enzymic interactions of paramyosin with actomyosin, *in vitro*, as a biochemical approach towards understanding the possible physiological relationships between these proteins. Szent-Györgyi and his colleagues have demonstrated that paramyosin forms the cores of thick filaments in molluscan muscles, which clearly indicates a structural role, and that rapid coprecipitation of molluscan paramyosin and actomyosin inhibits the latter's Mg^{2+}-ATPase (3). The authors suggested that paramyosin inhibits the ATPase by stabilizing the complex of actin and myosin, a possible explanation of the catch state of molluscan muscles in which considerable tension is generated without significant ATP hydrolysis. Conversely, Nonomura found no enzymatic differences between myosin-decorated paramyosin paracrystals and slowly precipitated myosin filaments (4). Such apparently discordant results arising from different experimental situations suggested to us that a more detailed examination of the functional properties of paramyosin was necessary.

Our studies emphasize paramyosin from the clam *Mercenaria mercenaria* and myosin from rabbit skeletal muscles because these well-characterized proteins may be prepared in both their native forms and as proteolytically cleaved segments (5, 6). The study of the cleaved segments permits specification of the molecular substructures within myosin and paramyosin whose interaction leads to the inhibition of actomyosin ATPase. The effects of actin and paramyosin upon actomyosin activity appear competitive with one another on the basis of reciprocal plots of activity and F-actin concentration. Thus, the binding of paramyosin to the rodlike segments of myosin in rapidly precipitated cofilaments decreases the apparent affinity of myosin for F-actin, whereas paramyosin does not alter the enzymatic properties of myosin in slowly formed cofilaments. The apparent discrepancy between previous reports (3, 4) concerning the enzymic properties of myosin–paramyosin cofilaments is explained by the different conditions for precipitation and the concentrations of F-actin that were used. Our results suggest that instead of stabilizing actomyosin interactions, paramyosin hinders the association of actin and myosin under specific conditions.

Abbreviations: LMM, light meromyosin; HMM, heavy meromyosin; HMM SF1, subfragment 1.

MATERIALS AND METHODS

Paramyosin composed of 94,000 (cleaved) and 105,000 (native) dalton monomers was purified from white and red adductor muscles of *Mercenaria mercenaria* by method II of Stafford and Yphantis in the presence of 1 mM phenylmethylsulfonylfluoride and 10 mM EDTA (5). Single muscles that were carefully dissected were immediately homogenized in a Sorvall Omnimixer (DuPont) and centrifuged to separate myofibrils quickly from the muscle supernatant. This modification was crucial to the preparation of the pure native species. Myosin was purified from the hind leg and back muscles of rabbits and from the adductor muscles of *Mercenaria mercenaria* (3, 7). Actin was purified from rabbit (8) and clam (3) muscles. Either F-actin was used interchangeably, as no significant difference in their properties was detected in our experiments. Light meromyosin (LMM), heavy meromyosin (HMM), and heavy meromyosin, subfragment 1 (HMM SF1) were prepared from rabbit myosin. HMM SF1 was purified from a papain digestion whereas the other two segments were the result of tryptic cleavage. Protein concentrations were determined by a modification of Lowry's procedure (9). The purity and composition of these proteins, and their fragments and filaments resulting from precipitation, was determined by sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (10). Ratios of proteins in various filaments were determined by densitometry of gels stained with Coomassie brilliant blue.

Mg^{2+}-ATPase activities were measured in a pH stat (Radiometer) under conditions indicated in *Results*, or by the radiochemical method of Spudich (11), which uses [γ-^{32}P]ATP. Reactions were initiated by the addition of ATP except as noted in the *text*. Actomyosin ATPase values refer to the specific enzymatic activity with respect to total myosin present. The intrinsic myosin ATPase activity was subtracted from the total ATPase activities in the calculation of reciprocal plots (12–14).

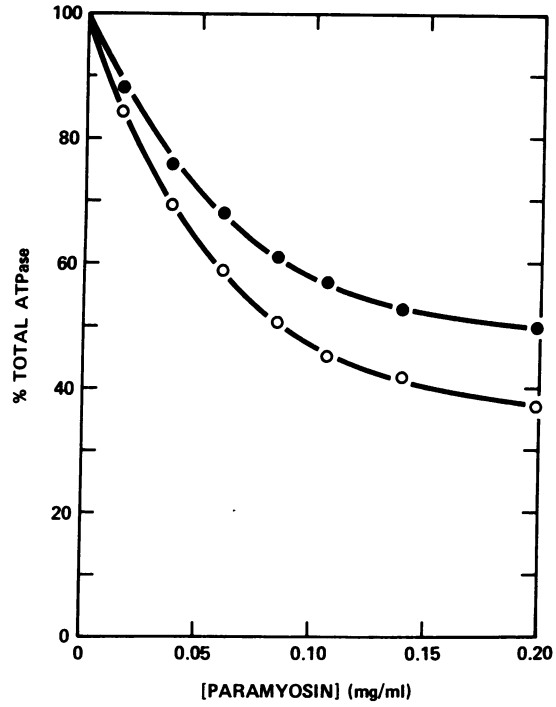

FIG. 1. Comparison of clam and rabbit myosins. Assays were performed by the pH stat method at pH 7.4. Reaction conditions were 0.1 mg·ml^{-1} myosin, 0.1 mg·ml^{-1} clam F-actin, 30 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.75 mM ATP. One hundred percent activity (no paramyosin present) was 0.33 and 0.28 μmol·min^{-1}·mg^{-1} for rabbit and clam myosins, respectively. Cleaved paramyosin was used. ○, Rabbit myosin; ●, clam myosin.

The maintenance of KCl concentration in the range 20–30 mM was necessary for obtaining the observed myosin ATPase activities. The rate of stirring during the rapid coprecipitation of myosin and paramyosin and the ensuing ATPase reaction was not critical. Rapid mechanical mixing in the pH stat and manual agitation in the radiochemical assay procedure produced equivalent results.

RESULTS

Clam and Rabbit Myosin Are Similarly Inhibited. Rabbit skeletal muscle myosin and its proteolytically cleaved fragments, HMM, HMM SF1, and LMM, are significantly more stable over the time required for our experiments than molluscan muscle myosin and its corresponding fragments, whose enzymic properties decay over a few days (15). In addition, rabbit myosin, HMM, and HMM SF1 have actin-activated Mg^{2+}-ATPase activities that are linear for over 20 min in our assays, whereas clam myosin does not. For these reasons, both myosins are compared in their inhibition by paramyosin so as to permit use of the rabbit proteins in further experiments. Rabbit and clam myosin, when mixed with 94,000 dalton clam paramyosin at 0.6 M KCl and then pipetted into the stirred reaction solution, are very similarly inhibited at the same concentrations of the paramyosin (Fig. 1). Each point represents a separate mixture of either myosin and paramyosin so precipitated to which F-actin and ATP are subsequently added for measurement of the indicated activities. The hyperbolic nature of this inhibition as a function of paramyosin concentration is qualitatively and quantitatively distinct from the apparently cooperative and complete inhibition of clam myosin by paramyosin previously reported (3). The intrinsic Ca^{2+}- and Mg^{2+}-ATPase activities of either myosin are not affected by paramyosin under these conditions.

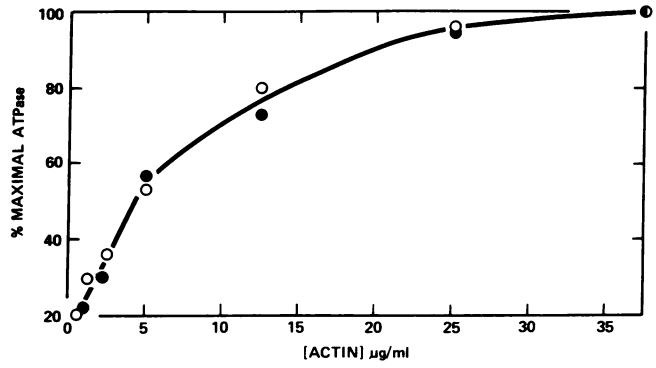

FIG. 2. Enzymatic activity of slowly precipitated filaments. ATPase activities were measured in the pH stat at pH 7.4. Myosin or myosin paramyosin mixtures at individual concentrations of 0.5 mg·ml^{-1} were dialyzed overnight at 4° against 0.1 M KCl, 10 mM $MgCl_2$, 10 mM potassium phosphate (pH 6.5). The filaments were collected by centrifugation and resuspended in 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 mM KCl, and 1 mM ATP. ATPase velocities were normalized to 0.89 and 0.73 μmol·min^{-1}·mg^{-1} for myosin and mixed filaments, respectively, at maximum rabbit F-actin concentrations. Indicated values are the average of duplicate measurements. ○, Rabbit myosin filaments; ●, rabbit myosin: cleaved clam paramyosin; 1:1.7 by weight. The amount of myosin was 95 μg in the pure myosin filaments and 90 μg in the mixed filaments. The total reaction volume in all cases was 2.0 ml.

Rapid Coprecipitation Is Required for Enzymic Inhibition. Rabbit myosin and clam paramyosin, after being mixed together at 0.6 M KCl and diluted into a stirring reaction solution to give 30 mM KCl, form a particulate suspension, as they do individually. The precipitated mixture hydrolyzes ATP at 0.165 μmol·min^{-1}·mg^{-1}, whereas an equivalent concentration of particulate myosin has an activity of 0.295 μmol·min^{-1}·mg^{-1} at 25°, pH 7.5 and 0.75 mM ATP. When myosin or paramyosin are allowed to precipitate alone and the other protein is added later, no inhibition is observed. Further, the inhibition is not affected by the order of addition of or by preincubation with F-actin or ATP, in contrast to the structural interactions of myosin and paramyosin observed under different conditions (4). Thus, rapid coprecipitation of myosin and paramyosin leads to the formation of a complex that is stable under our range of reaction conditions in which the actin-activated myosin ATPase is inhibited.

Slowly precipitated myosin–paramyosin filaments and myosin filaments have a qualitatively similar dependence of their Mg^{2+}-ATPase activities as a function of the concentration of F-actin added in the reaction mixture (Fig. 2). Similar results are obtained at both 1:0.95 and 1:1.7 ratios (determined by gel densitometry) of myosin to cleaved paramyosin within the filaments. The activities are normalized to the maximal rates obtained with each kind of filament, as the absolute amounts of each protein in the filaments could not be determined. Either kind of slowly formed filament shows actin-activated activities enhanced over those of similarly composed rapidly precipitated filaments.

Myosin and paramyosin that are coprecipitated rapidly or slowly in the same way as for the mixtures used in the ATPase assays yield filaments with apparent morphological differences. Rapidly made cofilaments are of 10–20 nm diameter and show no evidence of axial periodicity or of protruding headlike structures. Slowly formed cofilaments demonstrate an underlying axial periodicity, usually of 14.5 nm, partially obscured by globular structures at the surface (16).

The lack of any enzymic inhibition in the slowly formed cofilaments suggests that coprecipitation alone is not sufficient

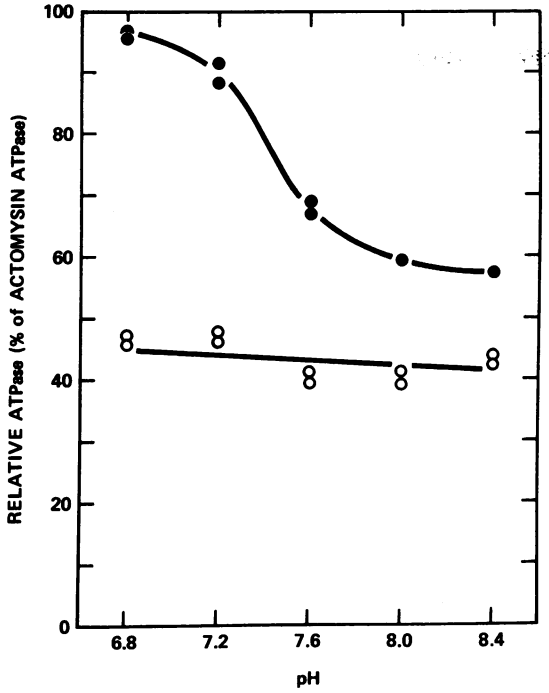

FIG. 3. pH dependence of inhibition by native and cleaved paramyosins. Assays were performed in the pH stat. Reaction conditions were 0.1 mg·ml^{-1} rabbit myosin, 0.1 mg·ml^{-1} paramyosin of either kind, 0.1 mg·ml^{-1} rabbit F-actin, 25 mm KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.75 mM ATP. 100% activity in the absence of paramyosin at pH 7.4 was 0.3 μmol ATP·min^{-1}·mg^{-1} for rabbit myosin at the above conditions. The activities in the presence of either paramyosin were normalized to that of actomyosin at each pH. ●, Cleaved paramyosin; ○, native paramyosin.

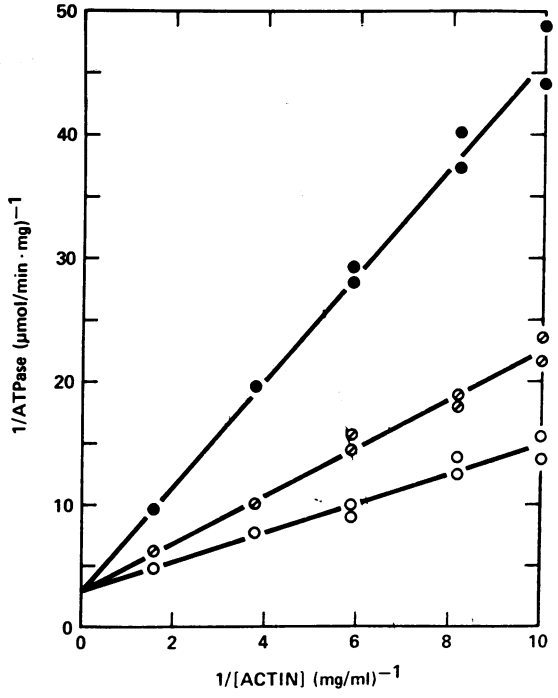

FIG. 4. Inhibition by native and cleaved paramyosins as a function of actin concentration. Assays were performed by the [γ-^{32}P]ATP method. Reaction conditions were rabbit F-actin as indicated, 0.15 mg·ml^{-1} paramyosin of either kind, 0.1 mg·ml^{-1} rabbit myosin, 30 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 25 mM Tris·HCl at pH 7.5, and 0.75 mM ATP. ○, No paramyosin; ⊘, cleaved paramyosin; ●, native paramyosin.

for the inhibition seen with the rapidly formed structures. Different interactions between myosin and paramyosin within cofilaments formed under the two conditions might explain the difference in behavior of these structures.

LMM Contains Sites Necessary for the Inhibition. Paramyosin has no effect upon the actin-activated ATPase of rabbit HMM or HMM SF1 at 0.8 mg·ml^{-1}, which reduces the activity of a mixture containing 0.15 mg·ml^{-1} F-actin and 0.1 mg·ml^{-1} myosin from 0.35 to 0.09 μmol·min^{-1}·ml^{-1} at 25°, pH 7.5 and 1.0 mM ATP. These experiments eliminate the possibility that under our conditions direct interaction between paramyosin and the ATPase sites of myosin or the myosin binding sites of F-actin is responsible for the inhibition. Rabbit LMM, on the other hand, antagonizes the inhibition of actomyosin ATPase by paramyosin after LMM, myosin, and paramyosin have been rapidly coprecipitated.

Native and Cleaved Paramyosins Differ Quantitatively in Their Inhibition. Paramyosins containing 105,000 dalton (native) and 94,000 dalton (cleaved) polypeptides may be isolated in the presence or absence of 10 mM EDTA and 1 mM phenylmethylsulfonylfluoride, respectively (5). The larger protein is presumably the native molecule, whereas the cleaved protein still retains many physical and structural properties. The two paramyosins exhibit a marked difference in inhibitory properties as a function of pH. Between pH 6.8 and 8.4 the native species shows no significant change in its effect when normalized to the ATPase of actomyosin alone under the same conditions. As shown in Fig. 3, the cleaved paramyosin inhibits only slightly at pH 6.8 and more markedly with increasing pH. This function approximates a simple acid-base titration with a pK of 7.3. The fact that the state of ionization of certain groups is critical for inhibition by cleaved paramyosin suggests that localized interactions might be necessary for this inhibition of myosin by paramyosin. The solubility of paramyosin does not significantly change over this pH range at this ionic strength (17). Therefore, the increased inhibition with more alkaline pH does not appear to be due to increased precipitability of cleaved paramyosin.

At pH 7.5, the effect of both paramyosins upon myosin ATPase as a function of F-actin concentration is compared to myosin alone in Fig. 4. All three myosin mixtures exhibit linear reciprocal plots with respect to F-actin concentration. This result suggests that the individual myosins within the different kinds of filaments behave independently of one another with respect to activation by F-actin. Extrapolation to infinite actin concentration shows no difference between paramyosin complexed with myosin and and myosin alone, which suggests competition between the effects of actin and paramyosin. The apparent V_{max} for these experiments is 0.34 μmol·min^{-1}·mg^{-1}. At all lower actin concentrations, native paramyosin is a better inhibitor than cleaved paramyosin on a molar or weight basis. This substantial quantitative difference due to the loss of about 10% of the paramyosin rod length suggests that specific interactions between myosin and paramyosin may be required for the inhibition.

Paramyosin Competes with Actin for Myosin. Actin and paramyosin appear to compete with one another for the activation and relative inhibition of myosin Mg^{2+}-ATPase, respectively. To resolve the mode of inhibition more fully, we have studied myosin ATPase activity as a function of both F-actin and native paramyosin concentrations. In addition, the effect of paramyosin upon the intrinsic Mg^{2+}-ATPase of myosin was investigated.

Double-reciprocal plots of ATPase as a function of actin

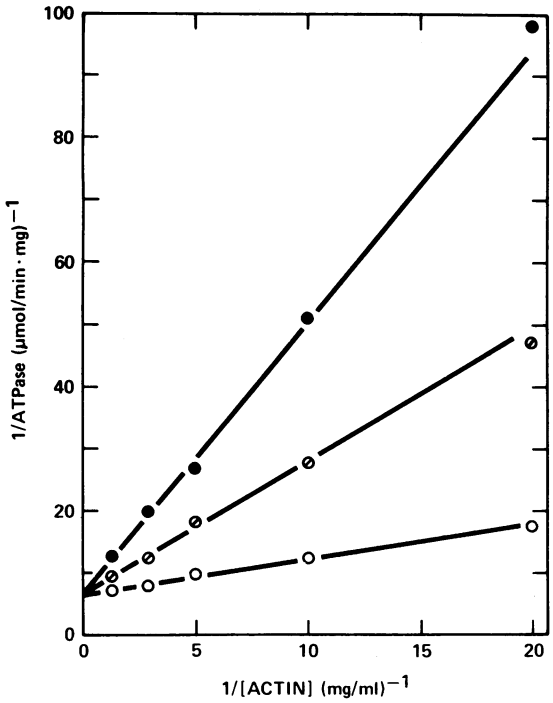

FIG. 5. Competitive kinetics of inhibition. Assays were performed by the pH stat method at pH 7.4. Reactions were initiated by the addition of rabbit F-actin. Reaction conditions were 0.1 mg·ml^{-1} rabbit myosin, 24 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.75 mM ATP. ○, No paramyosin; ⊘ and ●, 0.05 and 0.2 mg·ml^{-1} of native paramyosin, respectively.

concentration at different concentrations of native paramyosin (Fig. 5) confirm the competitive nature of the inhibition suggested in Fig. 4. It should be stressed that the linear reciprocal plots shown in the two figures were obtained by two different assay methods. Similar results by both assays have been obtained with different protein preparations. The absolute ATPase activities vary, but the linearity and the extrapolated intersection of the plots do not. The apparent V_{max} of the ATPase in all three cases was 0.33 μmol·min^{-1}·mg^{-1}. Therefore, paramyosin appears to decrease the apparent affinity of F-actin and myosin for one another. This conclusion is further supported by the observation that F-actin in a paramyosin-inhibited actomyosin mixture activates HMM SF1, whereas in the absence of paramyosin the same addition of HMM SF1 does not increase ATPase activity (Fig. 6). Under these conditions, paramyosin partially prevents physical association of myosin and F-actin molecules, as evidenced by the increased availability of actin sites. Paramyosin has no significant effect upon the enzymatic properties of myosin alone. Paramyosin concentrations sufficient to inhibit actin-activated myosin Mg^{2+}-ATPase significantly slightly increase the intrinsic activity. Thus, rapid coprecipitation of the two proteins does not alter directly the catalytic sites of myosin or prevent Mg^{2+}-ATP from binding to them.

A direct physical blocking of myosin ATPase sites in the rapidly coprecipitated filaments that prevents actomyosin association would be expected to lower the V_{max} for F-actin because such trapped myosin would not be able to associate with F-actin even at high actin concentrations. Preincubation with F-actin did not affect the inhibition of rapidly coprecipitated myosin and paramyosin (data not shown), which suggested that the latter proteins maintain a stable association under these reaction conditions. Paramyosin does not have any effects upon HMM or F-actin. Thus, the strictly competitive nature of the

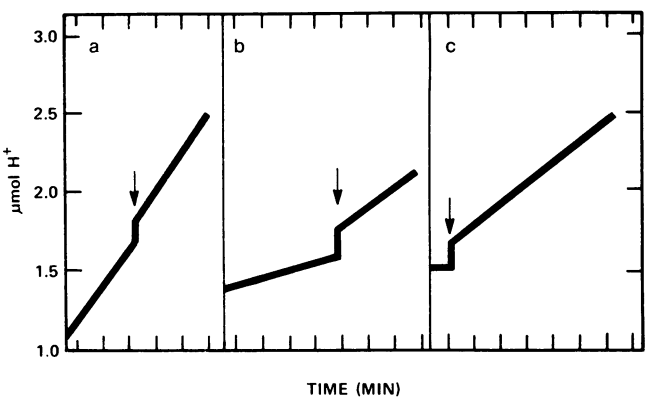

FIG. 6. Availability of F-actin during paramyosin inhibition. Assays were performed by the pH stat method at pH 7.5 and 20°. Reaction conditions where appropriate were 0.16 mg·ml^{-1} rabbit HMM SF1, 0.32 mg·ml^{-1} clam adductor paramyosin, 0.16 mg·ml^{-1} rabbit myosin, 0.07 mg·ml^{-1} rabbit F-actin, 24 mM KCl, 2 mM $MgCl_2$, and 1.0 mM ATP. The reaction volume was 5.0 ml. (a) HMM SF1 added to actomyosin. (b) HMM SF1 added to actomyosin–paramyosin. (c) HMM SF1 added to F-actin. The arrow denotes when HMM SF1 was added. The data were traced from actual pH stat records; the abscissa is marked in 1 min intervals. Note that F-actin was limiting in these experiments.

partial inhibition suggests that a change in some intrinsic properties of myosin may occur as a result of rapid coprecipitation with paramyosin.

DISCUSSION

Paramyosin is found in functionally and structurally diverse muscles from many invertebrate phyla, including molluscs (17), insects (2), and nematodes (1). In molluscan thick filaments, myosin binds to the surface of paramyosin-containing cores, probably as the result of bonding between the α-helical, coiled-coil regions of both molecules (3, 4, 18). Apparently discordant conclusions have been reach as to whether the interactions between myosin and paramyosin affect actomyosin association and thereby trigger the catch state of these muscles (3, 4).

The understanding of interactions between myosin fragments and F-actin has been enhanced by the measurement of Mg^{2+}-ATPase activities as a function of F-actin concentration (13, 14). The linearity of reciprocal plots of such measurements has permitted determination of apparent equilibrium constants for the association of F-actin and specific myosin fragments. We have obtained qualitatively similar relationships in the activity of rapidly precipitated myosin and myosin–paramyosin filaments as a function of added F-actin. The comparison of the linear reciprocal plots at different concentrations or with different forms of paramyosin suggests that paramyosin in a specific manner decreases the apparent affinity of myosin for F-actin. The increased availability of F-actin sites as monitored by the activation of added HMM SF1 in such reaction mixtures is consistent with this apparent competition. Instead of a physically competitive relationship, we propose that the association equilibrium constant for the reaction

$$\text{F-actin} + \text{Myosin} \cdot \text{ATP} \rightleftharpoons \text{F-actin} \cdot \text{Myosin} \cdot \text{ATP}$$

is lower in rapidly formed myosin–paramyosin cofilaments than in similarly formed myosin filaments.

The inhibition of actomyosin Mg^{2+}-ATPase is dependent upon the conditions under which myosin and paramyosin form cofilaments. The activities of slowly formed filaments containing myosin alone or complexed with paramyosin are similar

as a function of F-actin, in contrast to the case in the rapidly precipitated proteins. The enhanced maximal activity of the slowly formed structures and their activation at lower F-actin concentrations when compared to the rapidly precipitated filaments is not understood. A possible explanation might be that myosin in either kind of slowly formed filament more easily binds F-actin sites due to a more regular arrangement about the surface. The differences in activities between slowly and rapidly formed cofilaments of myosin and paramyosin show that coprecipitation, itself, is not the cause of the inhibition by paramyosin that we have observed.

The inhibition of actomyosin by paramyosin appears to have specific molecular requirements. The observed qualitative and quantitative differences between native and cleaved paramyosins with respect to their inhibitory properties support this hypothesis. The absence of any effects of paramyosin upon HMM, HMM SF1, or F-actin suggests that interactions between the rodlike portions of myosin and paramyosin are necessary for the inhibition. Abolition of inhibition by coprecipitation of LMM with myosin and paramyosin is consistent with such a proposal. The structural requirements for the inhibition as determined by our *in vitro* experiments under specific experimental conditions are qualitatively similar to known relationships between myosin and paramyosin in native thick filaments (3, 4). A possible model to explain our results is that interactions between additional rodlike portions of myosin, such as heavy meromyosin subfragment 2, and of paramyosin might be responsible for the decreased affinity of the myosin for F-actin observed in rapidly but not slowly precipitated cofilaments and for the greater inhibition of native paramyosin than of the cleaved species. In muscle that contains paramyosin, a reversible equilibrium might exist between these facultative interaction sites on myosin and paramyosin rods, leading to states of either high or low affinity for F-actin. Our experimental conditions may trap the cofilament in the low affinity state.

Although the physiological significance of the inhibition has not been determined, previous suggestions that paramyosin stabilizes actomyosin association during the catch state (3) are not consistent with the competitive effects of paramyosin and F-actin in the presence of ATP or the increased availability of F-actin in inhibited actomyosin mixtures that we have observed. As paramyosins from diverse invertebrate sources, including nematodes (H. E. Harris and H. F. Epstein, unpublished results), show this inhibition, whereas catch has not been observed in these muscles, the role of paramyosin and its association with myosin *in vivo* may be related to other functions. For example, paramyosin might be involved in the relaxation from catch and other special states of invertebrate muscles. Enzymatic studies of different kinds of myosin–paramyosin cofilaments, both natural and reconstituted, can serve as useful experimental tests of such physiological hypotheses.

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