Activation of the Adenosine Triphosphatase of *Limulus polyphemus* Actomyosin by Tropomyosin

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ABSTRACT Purified actin does not stimulate the adenosine triphosphatase (ATPase) activity of *Limulus* myosin greatly. The ATPase activity of such reconstituted preparations is only about one-fourth the ATPase of myofibrils or of natural actomyosin. Actin preparations containing tropomyosin, however, activate *Limulus* myosin fully. Both the tropomyosin and the actin preparations appear to be pure when tested by different techniques. Tropomyosin combines with actin but not with myosin and full activation is reached at a tropomyosin-to-actin ratio likely to be present in muscle. Tropomyosin and actin of several different animals stimulate the ATPase of *Limulus* myosin. Tropomyosin, however, is not required for the ATPases of scallop and rabbit myosin which are fully activated by pure actin alone. Evidence is presented that *Limulus* myosin, in the presence of ATP at low ionic strength, has a higher affinity for actin modified by tropomyosin than for pure actin.

INTRODUCTION

Contraction is the result of an interaction between actin, myosin, and adenosine triphosphate (ATP) (Szent-Györgyi, 1951). Nevertheless, the properties of actomyosin are readily modified by other proteins. Physiologically, the most important modifier is the troponin-tropomyosin complex which enables muscle to relax by blocking interaction between actin and myosin (Ebashi and Endo, 1968). In vitro these proteins inhibit the actin-activated adenosine triphosphatase (ATPase) of myosin, provided calcium is absent (Weber and Herz, 1963). An opposite effect, namely activation, has been occasionally associated with crude relaxing factor preparations (Katz, 1964; Hartshorne and Mueller, 1969; Schaub et al., 1967). While this activation is variable, it can amount to about 50–100% using rabbit actomyosin (Greaser and Gergely, 1971) or a rabbit acto–heavy meromyosin system (Spudich and Watt, 1971). The component responsible for activation has not been unambiguously identified, and its relationship to the relaxing system is not

clear. According to Schaub et al. (1967), activation of ATPase does not require the entire relaxing system; tropomyosin alone, however, is not effective.

In this paper, we report that the actin-activated ATPase of the myosin of *Limulus polyphemus* is strongly enhanced by tropomyosin. This activation does not depend on the source of tropomyosin, and is specifically shown only by *Limulus* myosin.

MATERIALS AND METHODS

A. *Preparations*

All proteins were prepared in the cold. Rabbit actin, free of tropomyosin contamination, was prepared as previously described (Kendrick-Jones et al., 1970). Rabbit and chicken tropomyosins were prepared by the procedure of Bailey (1948) with an additional purification step consisting of precipitating the tropomyosin from 5 mM tris(hydroxymethyl)amino methane (Tris), pH 8.0, by dialysis against 50 mM $MgCl_2$–5 mM Tris, pH 8.0, and redissolving the precipitate in 5 mM Tris, pH 8.0. The chicken tropomyosin was given to us by Dr. Sarah Hitchcock. Thin filaments from *Mercenaria mercenaria* and *Aequipecten* were prepared as described previously (Szent-Györgyi, Cohen, and Kendrick-Jones, 1971; Kendrick-Jones et al., 1970). Paramyosin of *Aequipecten* smooth adductor muscle was prepared according to Johnson et al. (1959). A crude preparation of troponin-tropomyosin relaxing proteins of rabbit was made according to Ebashi and Ebashi (1964) and was copolymerized with G-actin from rabbit.

LIMULUS MYOFIBRILS Washed leg, tail, and body muscles were dissected and rinsed with a solution of 50 mM NaCl, 1 mM $MgCl_2$, and 5 mM phosphate buffer, pH 7.4, and then homogenized in the same solution in a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) for 3–5 sec at full speed. The homogenate was sedimented at 5000 *g* for 5 min, dispersed manually, and resedimented three times in the same solution.

ACTOMYOSIN The washed myofibrils were resuspended at a concentration of about 5–10 mg/ml in about 300 ml of a solution consisting of 5 mM phosphate buffer, pH 6.0, and 5 mM ATP, pH 6.0, and rehomogenized for 3–5 sec. The homogenate was brought to a final volume of 0.4 M NaCl with 4 M NaCl and stirred for 5 min. The suspension was sedimented for 30 min at 80,000 *g*. The supernatant was diluted with 12 vol of 5 mM phosphate buffer, pH 6.0, the precipitate was collected by centrifugation, and redissolved in 0.6 M NaCl–5 mM phosphate, pH 7.0, and then this step was repeated.

MYOSIN Myosin was prepared from the crude actomyosin extract by differential centrifugation in the presence of ATP (Weber, 1956). The actomyosin solution was brought to 0.6 M NaCl and 10 mM $MgCl_2$, an additional 5 mM ATP was added, and the pH was adjusted to 7.0 by adding 0.5 M NaOH dropwise. The solution was then sedimented for 5 hr at 80,000 *g* to remove part of the actin content and the myosin was precipitated by dialysis against 12 vol of 5 mM phosphate buffer, pH 6.0, and adjusted to pH 6.0 with dilute HCl. The precipitate was collected by centrif-

ugation and dissolved in about 100 ml of 0.6 M NaCl, 5 mM phosphate buffer, pH 7.0, and 5 mM ATP.

To remove the remaining actin contamination, the solution was sedimented for 12 hr at 100,000 *g*. The supernatant was collected and reprecipitated as before, redissolved in 100 ml of 0.6 M NaCl, 5 mM phosphate buffer, pH 7.0, without ATP, and sedimented 12 hr at 100,000 *g*. The supernatant containing purified myosin was collected, reprecipitated, and redissolved in 0.6 M NaCl–5 mM phosphate buffer, pH 7.0.

LIMULUS THIN FILAMENTS (*a*) Long bundles of the muscle connecting the abdomen with the cephalothorax were tied to sticks at rest or slightly stretched length and were placed in 50% glycerol-water overnight.

(*b*) The bundles were teased into millimeter bundles while still attached to the sticks and placed in a room-temperature solution of 0.1 M KCl, 5 mM phosphate, 3 mM $MgCl_2$, 3 mM ethylenediaminetetraacetate (EDTA), pH 7.0, for 10 min.

(*c*) Thin filaments were then prepared by the procedure used to make thin filaments from the striated adductor of *Aequipecten irradians* (Kendrick-Jones et al., 1970).

RABBIT F-ACTIN TROPOMYOSIN COMPLEX Actin and tropomyosin were mixed in a weight ratio of 2 to 1, respectively, in a solution containing 50 mM NaCl, 1 mM $MgCl_2$, and 5 mM imidazole-HCl, pH 7.0, at an approximate concentration of 1 mg actin/ml and centrifuged at 100,000 *g* for 3 hr. The pellets were rinsed and suspended in the same solution with the aid of a Teflon homogenizer. Centrifugation and resuspension were repeated.

B. *Methods*

The Mg-activated ATPase of actomyosin and the Ca-activated ATPase of pure myosin were measured using the same procedure and assay solutions as previously described (Kendrick-Jones et al., 1970). The final salt concentration used in the assay medium was 25–30 mM.

Viscometry and protein determinations were performed as previously stated (Szent-Györgyi, Cohen, and Kendrick-Jones, 1971). Sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis was performed using Coomassie blue as a stain, according to Weber and Osborne (1969). Sedimentation velocity experiments were performed at 11.5°C in a Spinco Model E Ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) with a Schlieren optical system.

RESULTS

Limulus myosin is only slightly activated by purified actin preparations which do not contain tropomyosin. Myofibrils and natural actomyosin preparations have considerably higher ATPase activities (0.06–0.1 μmole ATP/min per mg) (see de Villafranca, 1966; de Villafranca and Campbell, 1969; Bárány, 1967) than actomyosins prepared from purified *Limulus* myosin and pure rabbit actin (0.02 μmole/min per mg) (Table I). The low ATPase activity of reconstituted actomyosin does not increase when the actin concentration is doubled (2 g actin to 3 g myosin) and cannot be due to the lack of suffi-

TABLE I

ATPASE ACTIVITIES OF *LIMULUS* PREPARATIONS

	No. of preparations	μmole phosphate liberated/min per mg*
Limulus myofibrils	4	0.065
Limulus actomyosin	3	0.066
Limulus myosin (pure)	5	<0.01
Limulus myosin and rabbit actin	5	0.018
Limulus myosin and *Limulus* thin filaments	2	0.081
Limulus myosin and *Mercenaria* thin filaments	2	0.085
Limulus myosin and *Aequipecten* thin filaments‡	1	0.048
Limulus myosin and rabbit actin and rabbit tropomyosin	5	0.086

* ATPase activities of myofibrils and actomyosin were calculated in terms of total protein present; the other values are in terms of myosin present. Myosin and actin were mixed in a 3 to 1 mg ratio and myosin and thin filaments in a 2.2 to 1 mg ratio. In two experiments larger amounts of actin were also tested (myosin-to-actin ratio was 3:2 w/w), both in the presence and in the absence of tropomyosin. ATPase activities were the same as those obtained with lower actin concentrations.

‡ Scallop thin filaments lose about half of their tropomyosin component during preparation.

cient amounts of actin. The slow ATPase of reconstituted actomyosin is not caused by the inactivation of the myosin component during purification, since the same myosin preparations when activated by actin added in the form of native thin filaments hydrolyze ATP at rates similar to those of untreated myofibrils and natural actomyosins (Table I). Thin filaments thus, in contrast to purified actin preparations, activate *Limulus* myosin fully.

Thin filaments of *Limulus* contain a number of components as judged by SDS acrylamide gel electrophoresis (Fig. 1). Two of the bands correspond to actin and tropomyosin. The proteins responsible for the calcium regulation of *Limulus* muscle are also associated with the thin filaments since the thin filaments bind calcium and the actomyosins formed by mixing the thin filaments with rabbit or *Limulus* myosin require the presence of small amounts of calcium for ATPase activity. Some of the bands seen probably represent components of the *Limulus* relaxing system. Any of these components may be responsible for the greater activating effect of the thin filaments on the ATPase activity of *Limulus* myosin.

Activation, however, is not restricted to *Limulus* thin filaments and a full activation can be achieved by the addition of both purified rabbit actin and rabbit tropomyosin (Table I). Maximum activation is reached when about 1.5 g of actin-tropomyosin complex is added to 3 g of myosin and the activation of ATPase remains the same at higher actin-tropomyosin concentrations.

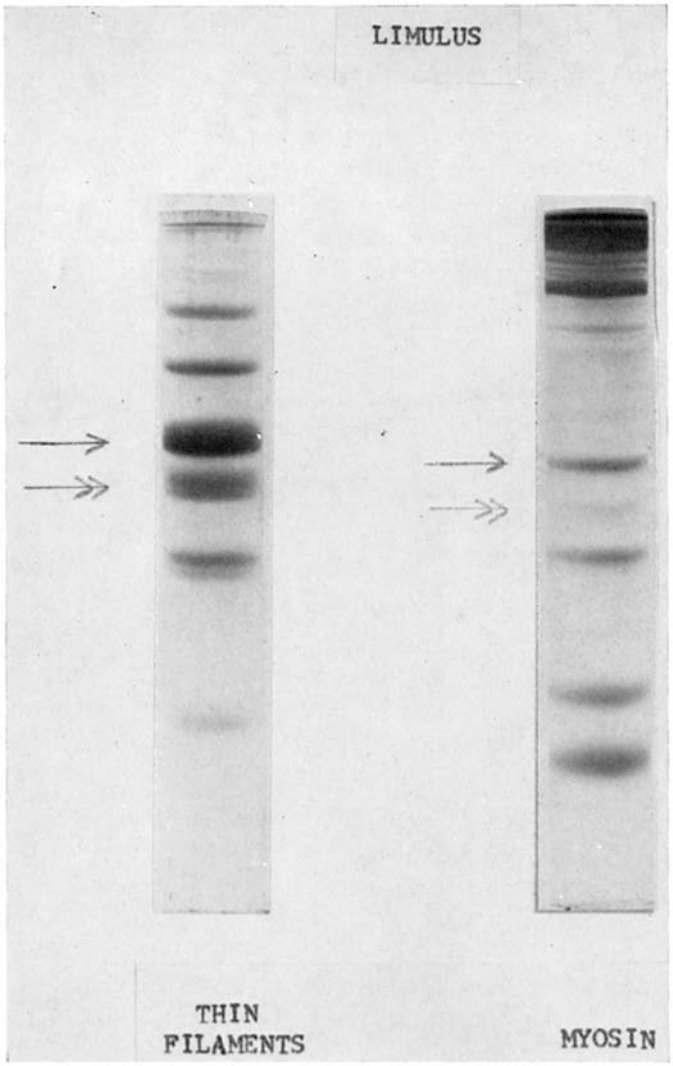

FIGURE 1. SDS gel electrophoresis patterns of *Limulus* thin filaments and myosin preparations. → actin; →» tropomyosin.

The actin and tropomyosin preparations used were free of troponin contaminations and the activation of the ATPase does not depend on the presence or the absence of calcium ions as indicated by the lack of effect of ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetate (EGTA). Both preparations behave as single components in SDS acrylamide electrophoresis (Fig. 2). The tropomyosin preparation chromatographs as a single component in diethylaminoethyl (DEAE) cellulose. Precipitation by trichloroacetic acid from 1 M NaCl, incubation in 0.1 M HCl–1.0 M NaCl, or repeated

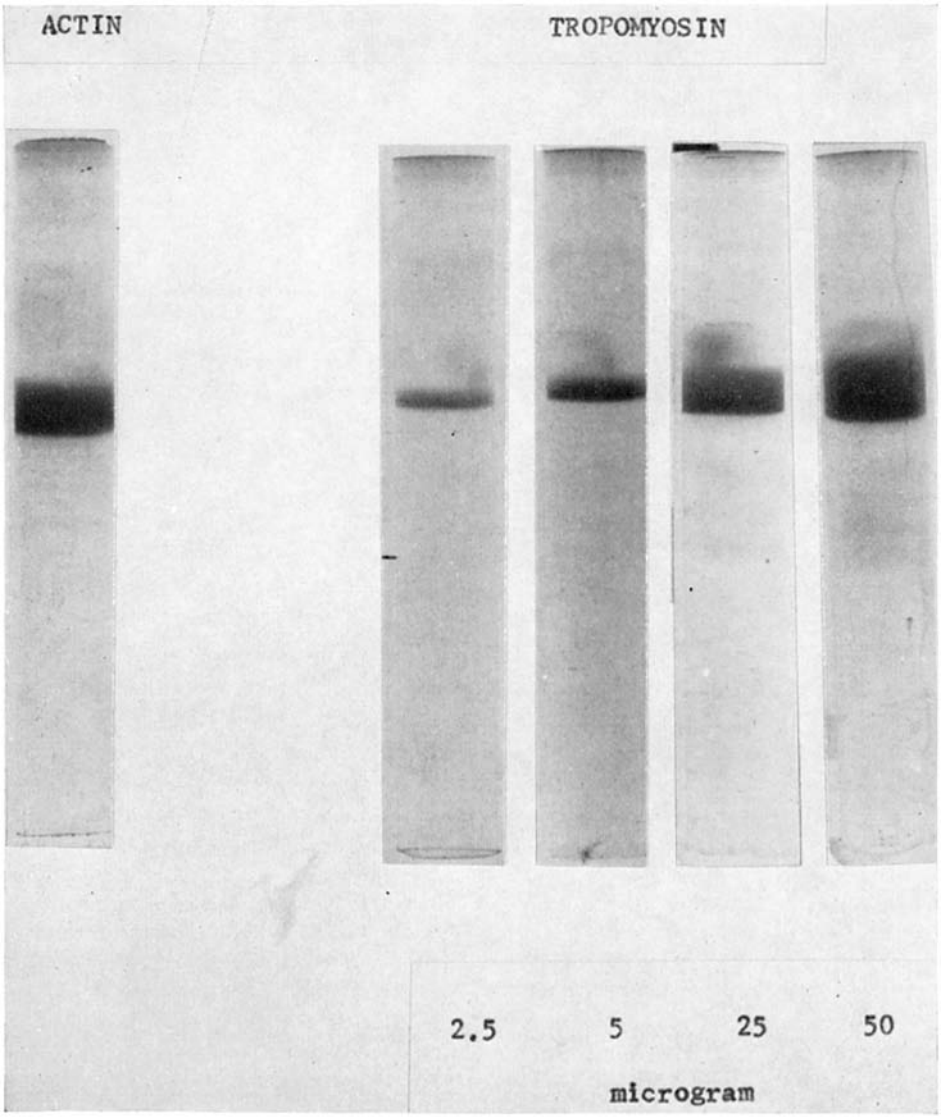

FIGURE 2. SDS gel electrophoresis patterns of rabbit actin and rabbit tropomyosin preparations. Both actin and tropomyosin preparations appear as single bands. Special procedures were followed for the electrophoresis of rabbit tropomyosin preparations which otherwise show a diffuse trailing edge. Tropomyosin in a concentration of about 0.1% was boiled for 10 min in 5% SDS, 2% β-mercaptoethanol, 10 mM phosphate, pH 7.2, and was dialyzed against 0.5% SDS, 2% β-mercaptoethanol, and 10 mM phosphate, pH 7.2, for 12 hr at room temperature. 10–20% glycerol was added to the mixture and it was run in a buffer consisting of 0.2% β-mercaptoethanol, 0.5% SDS, and 100 mM phosphate, pH 7.2, on gels prepared normally and equilibrated with the buffer for 2 hr or longer before the addition of the protein.

freezing and thawing does not lower the activating effect of the tropomyosin preparation. Since tropomyosin is not denatured by such treatments, these results are in agreement with the assumption that tropomyosin is the activator of *Limulus* actomyosin ATPase. Tropomyosins of other species also activate *Limulus* myosin. Chicken tropomyosin is as effective as rabbit (Fig. 3). This preparation travels as a single band in SDS acrylamide gel electrophoresis and was prepared and analyzed by Dr. S. Hitchcock. Thin filaments of

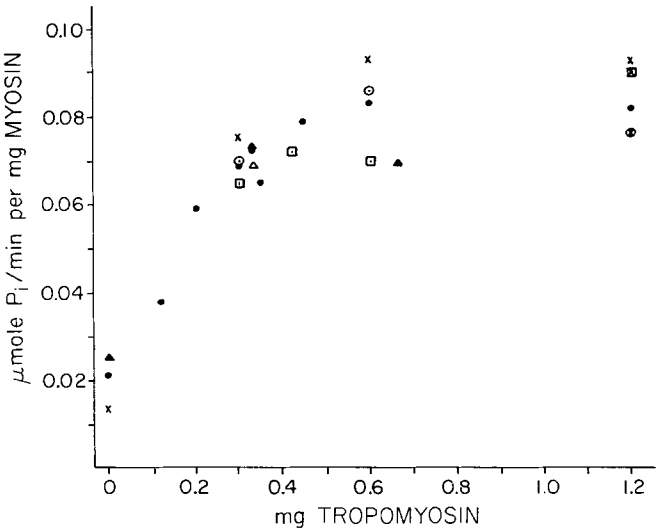

FIGURE 3. Dependence of the ATPase of the reconstituted *Limulus* actomyosin on tropomyosin concentration. Increasing amounts of tropomyosin were mixed with 1 mg rabbit actin in 50 mM NaCl–5 mM imidazole, pH 7.0. This mixture was then combined with 3 mg *Limulus* myosin dissolved in 0.6 M NaCl. The ATPase assay was performed in a solution of 25–30 mM NaCl, 1 mM Mg, and 0.5 mM ATP at pH 7.5. ●, x, and ▲, different *Limulus* myosin preparations with rabbit tropomyosin; △, *Limulus* myosin with chicken tropomyosin; ⊙, ⊗, *Limulus* myosin with acid treated tropomyosin; ⊡, ⊠, *Limulus* myosin with trichloroacetic acid (TCA)–treated tropomyosin.

Mercenaria and of scallop which contain tropomyosin but are lacking relaxing proteins (Szent-Györgyi, Lehman, and Kendrick-Jones, 1971) also activate *Limulus* myosin. The tropomyosin effect is specific, e.g., paramyosin, which is also an almost fully coiled α-helix (Cohen and Szent-Györgyi, 1957), does not activate, in fact, inhibits the ATPase activity of actomyosin.

Activation is proportional to tropomyosin concentration when tropomyosin is premixed with actin before addition of myosin (Fig. 3). Maximum activation is reached at the ratio of 1 mole of tropomyosin to 5–6 moles of actin (0.3 g tropomyosin/g actin) which is approximately the proportion in which these proteins are present in the thin filaments in vivo. The activation thus is unlikely to be due to a minor contaminant present in tropomyosin prepa-

rations. The actin-tropomyosin complex can be centrifuged without loss of activity, indicating that only material bound to actin is responsible for ATPase activation.

In contrast to the lack of species specificity of the actin and tropomyosin used, *Limulus* myosin appears to be unique among the various myosins investigated in requiring tropomyosin for ATPase activity. Actomyosins formed from rabbit or scallop myosin and pure actin have a comparatively high ATPase activity which is not stimulated any further by tropomyosin. Although *Limulus* myosin appears to be specific in requiring tropomyosin for its actin-activated ATPase, this requirement does not appear to be associated with an impurity present in *Limulus* myosin preparations. *Limulus* myosin sediments as a large, hypersharp peak with an additional slower component (Fig. 4). This slow component is likely to be paramyosin (de Villa-

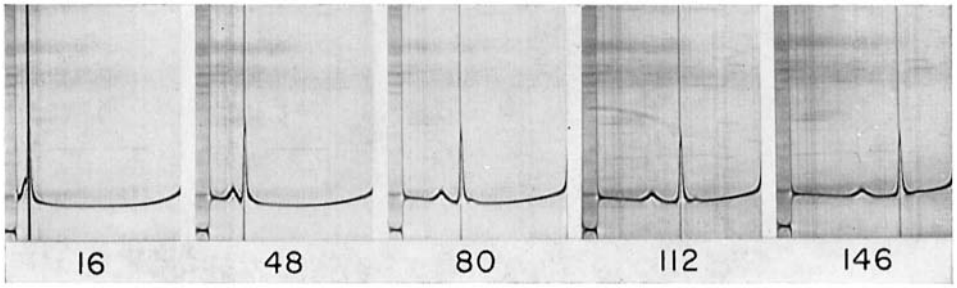

FIGURE 4. Sedimentation velocity patterns of *Limulus* myosin. Myosin was dissolved in 0.6 M NaCl–5 mM phosphate buffer, pH 7.0. Protein concentration, 3.7 mg/ml; bar angle, 70°; 11.5°C; 59,780 rpm. Sedimentation proceeds from left to right; number beneath each panel refers to time of sedimentation in minutes.

franca, 1971), since most of the trailing peak can be removed by centrifuging a concentrated myosin solution (~10 mg/ml) under conditions which selectively precipitate paramyosin: e.g., at pH 6.0 and an ionic strength of 0.35 for 15 min at 100,000 *g*. Removal of this component does not alter the enzymatic properties of myosin and the component is not required for ATPase activation.

We also have examined the possibility that tropomyosin may remove an inhibitor from *Limulus* myosin. *Limulus* myosin was mixed with rabbit tropomyosin in the ratio of 7.2 to 1 mg which is about 1 mole of myosin to 1 mole of tropomyosin. After preincubation in 0.6 M NaCl, pH 7.0, phosphate where both proteins are soluble, the myosin was precipitated by a 15-fold dilution with pH 6.5 5 mM phosphate buffer, whereas tropomyosin was still soluble. Any inhibitor which combined with tropomyosin would then be in the supernatant. The precipitated myosin was redissolved and its ATPase was tested with rabbit actin and was still found to require tropomyosin for activity. This result also confirms previous findings in rabbit that tropomyosin does

not combine with myosin under these conditions. Furthermore, tropomyosin does not increase the ATPase of *Limulus* myosin in the presence of 10 mM $CaCl_2$.

The relatively small activation of the myosin ATPase by pure actin may be the result of a low affinity between pure actin and myosin in the presence of ATP. Alternatively, an increased cross-bridge turnover rate in the presence of tropomyosin may also explain the activation of the ATPase. In the absence of ATP at high ionic strength, tropomyosin is not required for actin-myosin interaction and tropomyosin-free actin readily combines with *Limulus* myosin. The viscosity of *Limulus* myosin increases on addition of pure rabbit actin to the same extent as rabbit myosin does, and ATP addition causes a similar drop in the viscosity of both preparations. Nevertheless, evidence can be obtained that in the presence of ATP at low ionic strength formation of the actomyosin complex is enhanced by tropomyosin. If tropomyosin does not influence the combination of myosin and actin but increases the turnover rate, one would expect a competitive inhibition by pure actin when both tropomyosin-containing actin and pure actin are each added *in excess* of their stoichiometric combining ratio. No such inhibition was observed (Table II). It appears, therefore, that tropomyosin increases the apparent affinity between actin and myosin in the presence of ATP. In fact, when pure actin and myosin are premixed, before the addition of tropomyosin-containing actin preparations, the enzymatic activity starts slowly, then

TABLE II

COMPETITION BETWEEN PURE AND COMPLEXED ACTINS ON *LIMULUS* AND RABBIT MYOSIN

	Weight ratios of proteins used	Relative ATPase activities
Limulus myosin and actin	1.0:0.37	1.0
Limulus myosin and actin	1.0:0.5	1.1
Limulus myosin and actin-tropomyosin complex	1.0:0.37	3.0
Limulus myosin and actin-tropomyosin complex	1.0:0.5	3.1
Limulus myosin and actin and actin-tropomyosin complex	1.0:0.37:0.37	3.3
Limulus myosin and actin and actin-tropomyosin complex	1.0:0.5:0.5	3.0
Rabbit myosin and rabbit actin	1.0:0.5	1.0
Rabbit myosin and rabbit actin-tropomyosin-troponin complex	1.0:0.625	0.37
Rabbit myosin and rabbit actin-tropomyosin-troponin complex	1.0:0.93	0.37
Rabbit myosin and rabbit actin and rabbit actin-tropomyosin-troponin complex	1.0:0.5:0.65	1.05

accelerates and reaches maximum only after a few minutes. This demonstrates that actin filaments are not permanently associated with myosin filaments in the presence of ATP and that a slow equilibium is established between the myosin associated with pure actin and the myosin associated with the actin-tropomyosin complex. If myosin has a higher affinity for the tropomyosin-containing actin, it will detach from pure actin and preferentially combine with the actin-tropomyosin complex.

The validity of this approach can be further tested by analyzing the inhibitory effect of the troponin-tropomyosin system on the ATPase of rabbit actomyosin in the absence of calcium. In principle, this inhibition may also be due to a reduced cross-link formation between actin and myosin or to a reduced turnover of the cross-links. It has been reported that the troponin-tropomyosin system decreases the affinity of actin for heavy meromyosin and for subfragment 1 in the absence of calcium (Parker et al., 1970; Eisenberg and Kielley, 1970). The effect of troponin-tropomyosin on the affinity of actin for intact myosin has not been tested yet. We found that an actin preparation containing troponin and tropomyosin did not interfere with the ATPase activation of pure actin using rabbit myosin in the absence of calcium when both types of actins are added in excess of the stoichiometric amounts (Table II). This finding is in agreement with the interpretation that relaxing proteins block the interaction between actin and myosin.

It is noteworthy that modification of the ATPase of actomyosin by other proteins is achieved by changing the affinity of actin to myosin. Both the inhibition by the troponin-tropomyosin system of rabbit actomyosin or the activation of *Limulus* actomyosin by tropomyosin can be explained by modifying cross-link formation.

DISCUSSION

Pure tropomyosin combines only with actin and the activation of the ATPase of *Limulus* myosin by tropomyosin must be a result of this combination between actin and tropomyosin. This reaction is thus an additional instance in which the interaction between actin and myosin can be modified by altering only the actin component (Weber and Bremel, 1971). The activation phenomenon has analogous features to the inhibitory effect of the troponin-tropomyosin system on the ATPase activity of different actomyosin preparations. In both cases modification of actin leads to an altered ATPase activity, and in both cases one tropomyosin molecule can affect about seven actin monomers (Ebashi and Endo, 1968). The essential difference is that, for relaxation, components in addition to tropomyosin are also required. The altered enzymatic activity reflects some change in the actin filament which facilitates interaction between actin and myosin. The modifying action of tropomyosin on rabbit actin has been demonstrated by other means. Grant

(1967) found that an F-actin formed from adenosine diphosphate (ADP) containing G-actin is stabilized by tropomyosin and depolymerizes to a lesser extent at lower temperatures. Ishiwata and Fujime (1971) observed an increased rigidity of the actin chain in the presence of tropomyosin using laser scattering. *Limulus* myosin appears to be unusual since its actin-activated ATPase requires tropomyosin and thus it demonstrates enzymatically that actin can be modified by tropomyosin. Bremel (personal communication) has, however, observed that subfragment 1, obtained by papain digestion from rabbit myosin, has an actin-activated ATPase which is also considerably activated by tropomyosin at low ATP concentrations. Enhancement of actin-activated ATPase by tropomyosin may thus have a wider occurrence.

The activation of actomyosin ATPase by tropomyosin appears to be restricted to *Limulus* myosin, and it is not known which particular feature of the *Limulus* myosin molecule could account for this effect. The most variable features of different myosins are their light chains (Locker and Hagyard, 1967; Samaha et al., 1970; Lowey and Risby, 1971; Sarkar et al., 1971). The light chains of *Limulus* myosin are different from those of rabbit (Fig. 1) and it is possible that the requirement for tropomyosin reflects a particular constraint imposed by its light chains. In *Limulus* striated muscle, myosin is arranged in about 5–μ long filaments (de Villafranca and Philpott, 1961; Ikemoto and Kawaguti, 1967). Tropomyosin activation, however, does not appear to depend on a particular filamentous structure since actomyosin reconstituted by rapid precipitation behaves like myofibrils.

Activation of the ATPase by an additional protein may be ascribed to several different causes. An inhibitor of enzymatic activity may be neutralized; the affinity between actin and myosin in the presence of ATP may be altered; the turnover of the cross-bridges already formed may be accelerated, e.g., by enhancing the release of products of the enzyme. The activation of ATPase activity by tropomyosin is likely due to an enhancement of cross-link formation between actin and myosin. This result indicates that regulation of cross-bridge formation may not be restricted to inhibition. Depending on a particular myosin, actin may be modified by additional components favoring or inhibiting interactions. Positive control of actomyosin activity of this type may not necessarily be restricted to *Limulus* myosin alone.

Tropomyosin appears to have a multiplicity of functions. It is a required component of the relaxing system in most muscles. It may be a component of the Z disc in striated muscles. It is found in the thin filaments of all muscles studied so far and may be involved in the determination of their length. In *Limulus* muscle tropomyosin appears to be necessary both for enzymatic activity itself and for its regulation by the relaxing system.

Note Added in Proof We have been able to prepare subfragment 1 from *Limulus* myosin. Its ATPase rate, when mixed with rabbit actin, is also activated by rabbit tropomyosin.

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