Interaction of Actin and Myosin in the Absence and Presence of ATP

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Actin-myosin interaction, actomyosin ATPase, actin, tropomyosin-troponin

The binding between F-actin and myosin has been studied by analyzing the amount of radioactively labelled (by means of ^{14}C-NEM) F-actin and myosin in the formed unsoluble actomyosin pellet after centrifugation of the reaction mixture. In the absence of ATP, the amount of actin bound to myosin varies, depending on the amounts of actin and myosin present, between 0.18 mg actin/mg myosin (2 actin units per 1 myosin molecule) and about 2 mg actin/mg myosin (each actin fibril only uncompletely saturated with myosin). In 0.03 M KCl ATP decreases, if at all, the affinity of actin towards myosin only slightly; but less actin is bound to myosin in the presence of MgATP and in low ionic strength, indicating that myosin is now more densily distributed over fewer actin fibrils leaving the rest of fibrils free. For precipitation of the total amount of myosin (myosin alone is soluble in MgATP) incomplete saturation of myosin with actin suffices; obviously actin promotes filament formation of myosin. The activation of myosin ATPase by actin depends in a similar manner as actin binding does on actin concentration, hence the enzymatic interaction between actin and myosin is accompanied by true actin-myosin binding. An actin-tropomyosin-troponin preparation, whose reduced viscosity is lower than that of F-actin and which consists of about 30% actin, activates myosin ATPase to the same extent as F-actin does. It competes with F-actin for the same binding sites on myosin and can in the presence of MgATP be displaced from myosin by those preparations of F-actin which have a strong tendency to become fully saturated with myosin. The activation of myosin ATPase by actin-tropomyosin-troponin is reduced after tryptic digestion of actin-tropomyosin-troponin, which affects, according to SDS gel electrophoresis, mainly two components of troponin with molecular weights of about 32 000 and 25 000, respectively.

Introduction

It is generally assumed that during muscle contraction a cyclical forming and breaking of crossbridges between thick and thin filaments of muscle takes place. These crossbridges are formed by the HMM part of the myosin molecules of the thick filaments. Because actin and myosin form spontaneously actomyosin *in vitro* one has to assume that the formation of the bridges between the two filaments is thermodynamically spontaneous and that ATP is used to break formed bridges in order to prepare them for the next cycle. At high ionic strength, the actin-myosin dissociating effect of ATP prevails and gives rise to a drastic reduction of actomysin ATPase. Obviously, binding of actin to myosin is a basic process in muscular contraction. *In vitro* studies of the interaction of the two proteins may therefore provide information relevant to the understanding of muscular contraction. This paper investigates actin-myosin interaction by analyzing the amount and the actin-myosin-ratio of formed actomyosin after mixing pure F-actin and myosin. This has been done in the absence of ATP as well as in its presence, that is, under conditions which cause "superprecipitation" of the formed actomyosin complex ("contraction"). Binding of F-actin to myosin in the presence of ATP has been supplemented by studying the dependence of actomyosin ATPase on the concentration of actin.

The thin filaments of muscle, however, consist not only of actin but contain also the regulatory proteins tropomyosin and troponin which are responsible for the regulation of actin-myosin interaction by Ca^{++}-ions (for reviews see Ebashi and Endo [4]; Ebashi, Endo, and Ohtsuki [5]). It is therefore desirable to study not only the interaction between myosin and pure actin, but also the interaction between myosin and actin which contains the regulatory proteins. Since the exact quantitative composi-

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Unusual abbreviations: EGTA, Ethyleneglycol-2-(2-aminoethyl)-tetraacetic acid; NEM, *N*-ethylmaleimide; SDS, sodium dodecylsulfate; PPO, 2,5-diphenyloxazole; bis-MSB, *p*-bis-(*o*-methylstyryl)-benzene; HMM, heavy meromyosin; HMM-S_1, subfragment 1 of HMM; LMM, light meromyosin.

Enzymes: ATPase (ATP phosphohydrolase): EC 3.6.1.3; trypsin: EC 3.4.4.4.

tion of the tropomyosin-troponin complex as well as its relation to actin is still a matter of investigation (*cf.* Spudich and Watt[6]; Spudich *et al.*[7]; Ebashi *et al.*[8]; Greaser and Gergely[9]) we circumvented this uncertainty by using an actin-tropomyosin-troponin preparation which could easily and reproducibly be obtained as a whole and which showed all properties it should have (Ca-dependent activation of myosin ATPase at low ionic strength and in the presence of Mg). This preparation is called actin-tropomyosin-troponin in this paper.

Methods

Preparation of proteins

Myosin and F-actin

Myosin and F-actin are essentially prepared as described earlier (Dancker[1]). From rabbit skeletal muscle myosin was extracted with 0.6 M KCl, separated from actin by centrifuging in 5 mM $MgCl_2$, 5 mM ATP at 150 000 × *g*; remaining actomyosin (if any) was precipitated by reducing the KCl concentration to 0.25 M. F-actin was extracted from acetone muscle powder with ice-cold water, partially polymerized in 0.7 mM $MgCl_2$, pH 8.

Actin-tropomyosin-troponin

The search for a convenient actin-tropomyosin-troponin preparation started from the observation that sometimes, after centrifugation of polymerized actin, from the remaining supernatants actin preparations could be obtained that yielded together with myosin a highly Ca-dependent actomyosin ATPase. Obviously, if actin extracts contain the regulatory proteins tropomyosin and troponin a certain proportion of actin is kept in the supernatant. Therefore, in order to develop a reliable preparation method, it is necessary that sufficient tropomyosin-troponin has been extracted prior to ultracentrifugation. Actin and tropomyosin-troponin must have been in contact before centrifugation of polymerized actin. The following procedure was applied. Actin aceton powder was extracted for 45 min with distilled water at room temperature, then KCl was added to a final concentration of 0.8 M, the pH was adjusted to 7 with histidine buffer and extraction was continued for one more hour. The extract was filtered and centrifuged for 2 hours at 115 000 × *g* in order to precipitate the polymerized actin. To 100 ml of supernatant 30 g solid $(NH_4)_2SO_4$ were added. The precipitate was dissolved in water and dialyzed over night in the cold against weakly buffered solution (about 2 mM tris-maleate, pH 7.2). The preparation tended to become gelatinous, if it became more acid than pH 6.5.

Binding between actin and myosin

For this purpose F-actin, actin-tropomyosin-troponin or myosin were labelled with radioactive ^{14}C-NEM.

Preparation of radioactive F-actin

Radioactive NEM (final concentration 50 μM) was added to the water extract of acetone powder before polymerization of actin and ultracentrifugation. Unbound NEM was removed by depolymerizing and repolymerizing the F-actin in 0.1 M KCl.

Preparation of radioactive actin-tropomyosin-troponin

After ultracentrifugation of the acetone powder extract radioactive NEM was added to the supernatant to a final concentration of 1 μM 30 min before ammonium sulfate precipitation. Then the precipitate was dissolved in a large amount of water in order to dilute unbound NEM and precipitated once more with ammonium sulfate. Afterwards it was dialyzed as already described.

Preparation of radioactive myosin

Myosin (about 5 mg/ml) was incubated for 20 min at pH 7.2 with 1 μM NEM and then twice precipitated and dissolved by variation of ionic strength in order to remove unbound NEM.

Performance of the binding experiments

The principle of the binding experiments was to centrifuge down the formed actomyosin under condition under which the free labelled compounds were soluble. The radioactivity in the pellet was counted. When both actin and myosin should be measured each experiment was performed twice under indentical conditions with either labelled actin and unlabelled myosin or vice versa. The experiments were done at room temperature and in 10 ml assays, pH 7.2 (0.01 M tris-maleate buffer). The concentration of KCl was usually 0.03 M. When in the absence of ATP both actin and myosin should be determined, the ionic strength was raised to 0.25 M KCl, because at this ionic strength free myosin is soluble. In the presence of ATP the ionic strength was always low (0.03 M KCl) because otherwise the interaction between actin and myosin

is inhibited by ATP. ATP and $MgCl_2$ were both 1 mM or 2 mM.

Under all conditions control experiments were done with varying amounts of radioactive protein without its partner to check how much of unbound protein would precipitate during centrifugation. The fraction of precipitated protein was linearly related to the total protein concentration in the centrifuge tube and if this fraction was only small as it was in the case with actin and myosin at 0.25 M KCl or with actin in 0.03 M KCl, the following correction formula could be applied

$$c = (protein_t - b)/(1 - a);$$

c: Corrected amount of radioactive protein in the supernatant; b: Total amount of radioactive protein in the pellet; a: Amount of unbound radioactive protein which precipitated per 1 mg of free radioactive protein; $protein_t$: Total amount of added radioactive protein.

The actomyosin pellets were dissolved in hyamine and counted in a solution containing dioxane and ®omnifluor (98% PPO + 2% bis MSB) in a Packard Liquid Scintillation counter.

Determination of ATPase activity

ATPase activity was measured at room temperature under conditions optimal for the contractile actomyosin ATPase. 10 ml assays contained 0.03 M KCl, 0.02 M tris-maleate buffer, pH 7.2; 2 mM $MgCl_2$, 2 mM ATP and about 1.5 – 2.0 mg myosin, about 1 mg F-actin or 1.5 mg actin-tropomyosin-troponin. The measurements were started by adding the ATP; aliquots were taken at 1 and 4 min and given into trichloroacetic acid (final concentration about 2%). Inorganic phosphate was measured according to Rockstein and Herron [2].

Further experimental procedures

Tryptic digestion. Actin-tropomyosin-troponin was digested at a weight ratio trypsin : actin = 1 : 10 at pH 7.2. The reaction was stopped with the double amount (with respect to trypsin) of soybean trypsin inhibitor.

Polyacrylamide gel electrophoresis was performed according to Weber and Osborn [3] with the following modifications: Acrylamide concentration was 7.5%; SDS (final concentration 1%) was only used to dissolve the protein but was omitted from the gel (no differences were found between runs with and without SDS in the gel). Na-glycerol-1-phosphate buffer (pH 7.4, 0.05 M) was used as gel and electrode buffer.

Protein concentration was determined by the biuret method calibrated against Kjeldahl nitrogen determination.

Viscosity was measured at 23 °C with a viscometer with a spirally formed capillary. The flow time for water was about 25 sec.

Material. ATP was obtained from Waldhof (Mannheim, Germany) EGTA from Fluka (Buchs, Switzerland), NEM from Serva (Heidelberg, Germany). ^{14}C-labelled NEM was purchased from NEN Chemicals (Dreieichenhain, Germany). Trypsin and trypsin inhibitor were purchased from Serva (Heidelberg, Germany).

Results

Binding of F-actin to myosin in 0.25 M KCl and in the absence of ATP

Fig. 1 shows how much actin and myosin is found in the pellet when increasing amounts of actin are added to a constant amount of myosin under conditions (0.25 M KCl) under which only actomyosin but neither free actin nor free myosin are insoluble. The amount of the precipitated actin stops rising when 0.24 mg actin/mg myosin are bound and begins even to decline although the actin-myosin ratio in the pellet continues to rise up to 2 mg actin/mg myosin. Hence, the amount of myosin in the pellet decreases too. Obviously, most of the formed actomyosin becomes soluble, when it contains such a quantity of actin that the solubility properties of actin prevail. The critical ratio lies in the range of 2 mg actin/mg myosin, because, when this ratio is reached, only a minute amount of the formed actomyosin is still found in the pellet.

On the other hand, the myosin content of actomyosin increases when the amount of added myosin is increased. This is seen in Fig. 1 B (inset) which shows that finally a ratio of 0.18 mg actin/mg myosin is approached. This ratio has to be expected, if every two actin units of an actin fibril bind one myosin molecule (see Discussion).

Interaction of F-actin with myosin in the presence of ATP and low ionic strength

The experiments of Fig. 1 B compare actin-myosin binding in the absence and presence of 1 mM ATP and Mg and are performed at low ionic strength in order to provide optimal conditions for superprecipitation of actomyosin. In the absence of

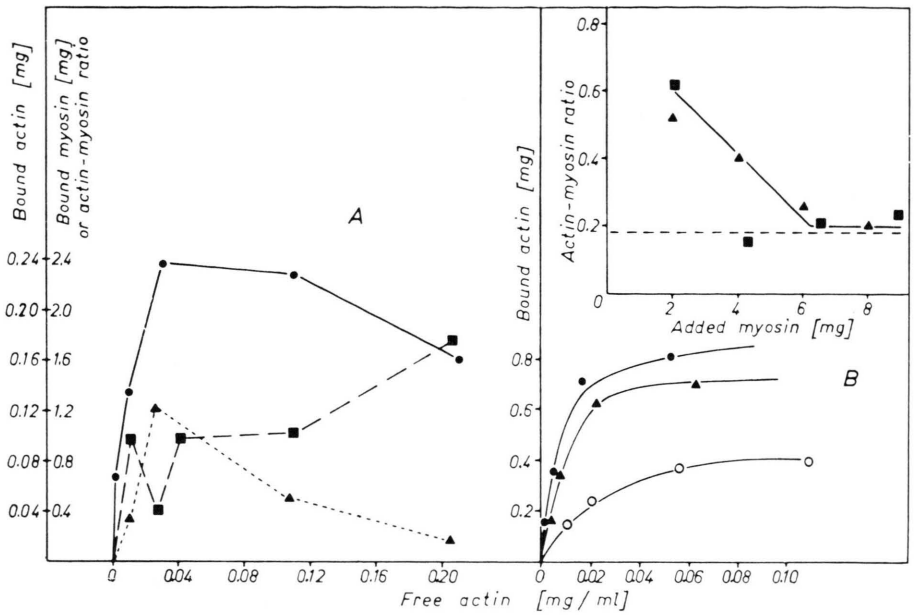

Fig. 1 A. Binding of F-actin to myosin and the actin-myosin ratio of actomyosin in 0.25 M KCl and without ATP. Each experiment was performed twofold with either radioactively labelled actin or myosin. ●—●: Amount of actin (mg/mg total myosin) in the pellet (left ordinate); ■ - - - ■: Atcin-myosin ratio (w/w) in the pellet (right ordinate); ▲- - -▲: Amount of myosin in the pellet (right ordinate). Total myosin: 2.2 mg in 10 ml assay.

Fig. 1 B. Comparison of actin-myosin binding in the presence and absence of polyphosphates and in 0.03 M KCl. ●—●: Control; ▲—▲: 1 mM Na-pyrophosphate, 1 mM $MgCl_2$; ○—○: 1 mM ATP, 1 mM $MgCl_2$; pH 7.2; 1.2 mg myosin. Inset: Actin-myosin ratio (w/w) in the actomyosin pellet as function of added myosin (0.25 M KCl, no ATP). Different symbols refer to two experiments. The dashed line indicates the actin-myosin ratio which corresponds to a stoichiometric ratio, if one myosin molecule binds two actin units. Note, that in the experiment of Fig. 1 B, in contrast to Fig. 1 A, the actomyosins of high actin content do not become soluble because the ionic strength is lower.

ATP the amount of actin, that appears in the pellet, approaches 1 mg/mg myosin. Inorganic pyrophosphate, which dissociates actomyosin at high ionic strength, changes the binding only slightly. In the presence of MgATP, however, less actin is found in the pellet, although the amount of bound myosin remains unchanged. This is shown in Table I which

Table I. Amount of myosin in the pellet and supernatant after centrifugation in the presence and absence of ATP and in low ionic strength.

Added actin	−ATP Myosin		+ATP Myosin	
	Super-natant	Pellet	Super-natant	Pellet
[mg]	[mg]	[mg]	[mg]	[mg]
0	0.058	0.742	0.730	0.070
0.25	0.044	0.750	0.043	0.757
0.50	0.060	0.740	0.075	0.725
1.0	0.049	0.751	0.075	0.725
1.5	0.039	0.761	0.066	0.734

To 0.8 mg myosin variable amounts of F-actin were added. 0.03 M KCl, 1 mM Mg = ATP; pH 7.2.

further shows that free myosin is soluble in the presence of ATP. Since the amount of myosin, which is part of actomyosin is not altered by ATP, one must assume, that myosin is now distributed over fewer actin fibrils which must be more completely saturated by myosin than before.

In fact, in the presence of MgATP very little actin is necessary to precipitate the whole amount of myosin. This can be seen from Fig. 2, which shows that the curve describing precipitation of myosin has already reached its maximum when the actin curve just begins to rise. In the absence of ATP and in 0.25 M KCl both curves do not deviate from each other (Fig. 1 A). In the absence of ATP and in 0.25 M KCl all precipitated actomyosin is rich in actin whereas in the presence of MgATP the first actomyosins, which appear in the pellet contain as little actin as about 0.05 mg/mg myosin. The total amount of myosin present is precipitated when about 0.1 mg actin/mg myosin is bound. This means that approximately 50% of the myosin molecules

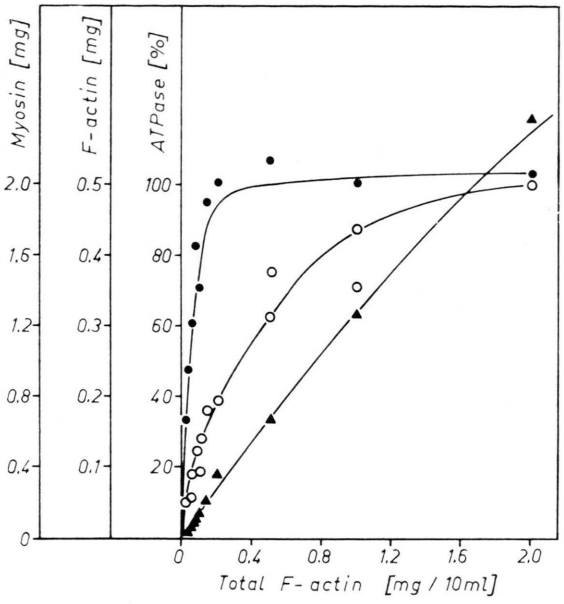

Fig. 2. Content of actin and myosin in the pellet and ATPase activity as function of total F-actin. 0.03 M KCl, 2 mM $MgCl_2$, 2 mM ATP, 2.2 mg myosin, pH 7.2. Abscissa: Amount of total F-actin in the assay (not the concentration of free actin as in the other Figs.). ●—●, Amount of precipitated myosin (mg); ▲—▲, amount of precipitated actin (mg/mg total myosin); ○—○, ATPase activity.

are combined with actin when all myosin is precipitated (100% myosin binding can be expected, when 0.18 mg actin/mg myosin is bound; see Discussion). This value agrees well with the ATPase activity, which has attained 40% of its maximal value when all myosin has been precipitated. When the stoichiometric ratio of about 0.2 mg actin/mg myosin is reached, ATPase activity, however, has attained only about 70% of its maximal value, indicating, that in this experiment not the whole actin, that is part of actomyosin and that should suffice to activate all myosin molecules interacts. In order to reach full ATPase activity a threefold excess of actin must be present in actomyosin. Accordingly, one has to assume that not all actin units of the F-actin fibrils used in this experiment are equally well suited for the interaction with myosin. Furthermore, the actin content of actomyosin continues to increase, even when the ATPase activity has reached its maximum. Hence, the binding curve diverges from that describing activation of ATPase activity.

ATPase and binding data are further compared in Table II. The data of this Table could be computed because binding curves as well as those curves

Table II. Activation of myosin ATPase by actin and binding of F-actin to myosin. The maximal rate of ATPase activity and the actin concentrations at which half-maximal ATPase activity occurs are extrapolated from double-reciprocal plots (1/added actin *versus* 1/ATPase activity). Maximal ATPase activity and actin concentration for half-maximal ATPase activity belong to one experiment, if listed in the same horizontal row. The respective values from binding studies are obtained by plotting 1/free F-actin *versus* 1/bound F-actin. Binding studies and ATPase measurements are obtained under identical conditions: 2 mM $MgCl_2$, 2 mM ATP, 0.03 M KCl, pH 7.2. The myosin content of the binding assays (10 ml) was about 2 mg, that of ATPase assays (10 ml) 1.5—2.0 mg.

ATPase measurements				Binding measurements	
F-actin		Actin-tropomyosin-troponin		F-actin	
Maximal rate of ATP-ase activity [μmoles Pi · mg myosin^{-1} · min^{-1}]	Actin-concentration at which ATPase-activity is half-maximal [mg/ml]	Maximal rate of ATPase-activity [μmoles Pi · mg myosin^{-1} · min^{-1}]	Concentration of A-TM-TP at which ATPase-activity is half-maximal [mg/ml]	Maximal amount of bound actin [mg/mg myosin]	Actin-concentration at which F-actin-binding is half-maximal [mg/ml]
0.23	0.011	0.21	0.070	0.40	0.055
0.42	0.033	0.48	0.076	0.45	—
0.34	0.040	0.31	0.061	0.17	0.041
0.31	0.023			0.17	0.030
0.45	0.020			0.30	0.078
0.25	0.028			0.25	0.012
0.44	0.035			0.50	0.050
0.50	0.015			0.31	0.048
0.22	0.036			0.72	0.033
mean: 0.35	0.027	0.33	0.07	0.36	0.043

relating activation of ATPase to actin concentration are generally hyperbolic, so that data of actin binding and of ATPase activation can both be plotted *versus* actin concentration in a linear manner analogous to the Lineweaver-Burk-plot or the Eadie-plot of enzyme kinetics (Webb [10]). This procedure has first been applied by Eisenberg and Moos [11] and Szentkiralyi and Oplatka [12] for analyzing activation of *H*-mero-myosin ATPase by actin, but has never been used to obtain the characteristic parameters of actomyosin ATPase. Table II summarizes data from experiments which have been performed at low ionic strength and in the presence of MgATP. The data of Table II indicate as does Fig. 2, that ATPase activity attains its half-maximal value at a lower actin concentration than does actin binding. The real difference may be even larger because in the ATPase experiments which were mostly performed without concomitant actin binding measurements the total actin concentration has been plotted rather than the true free actin concentration.

From the data presented in Table II it can be deduced that as an average 0.37 mg actin/mg myosin was maximally bound, indicating that under these conditions half of the actin units of the F-actin fibrils have been combined with myosin although in some experiments as that of Fig. 2, a larger amount of actin has eventually been bound. However, in all experiments in which actin binding has been compared in the absence and presence of ATP, we found more actin bound in the absence of ATP than in its presence.

Actin-tropomyosin-troponin activates myosin ATPase to the same extent as does F-actin but with a concentration of half-saturation which is about twice of that with F-actin, which can be explained by the lower actin content of actin-tropomyosin-troponin.

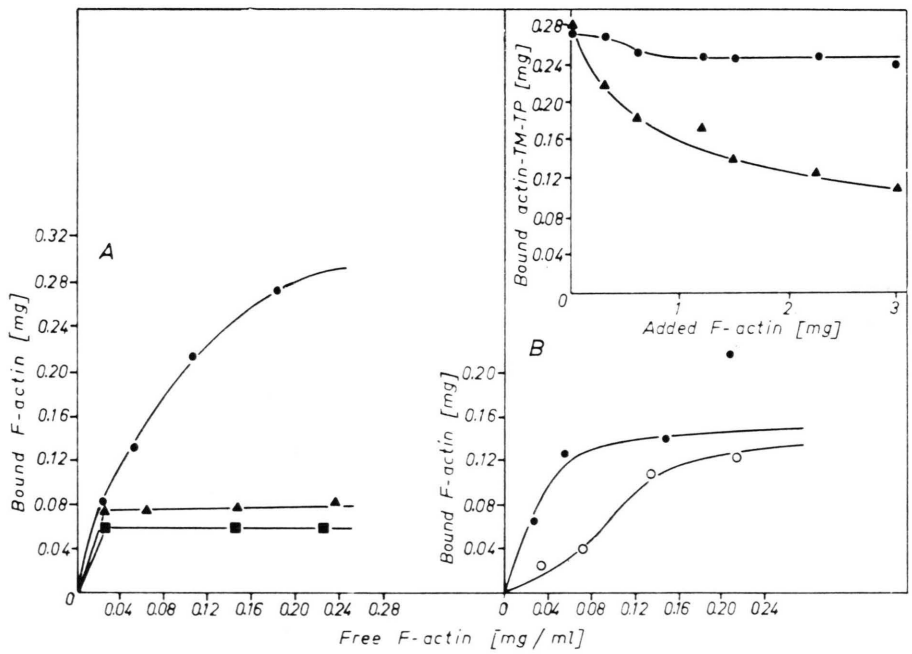

Fig. 3. Competition of binding between F-actin and actin-tropomyosin-troponin (0.03 M KCl, 2 mM ATP, 2 mM $MgCl_2$). A and B: Binding of F-actin to myosin. Actin-tropomyosin-troponin always added prior to F-actin. The binding assay (10 ml) contained 2 mg myosin and the following amounts of actin-tropomyosin-troponin. ●—●: No actin-tropomyosin-troponin; ▲—▲: 0.23 mg; ■—■: 0.34 mg; ○—○: 0.5 mg. A and B represent identically performed experiments with different results. Only in B F-actin has displaced actin-tropomyosin-troponin. Note the different amounts of bound F-actin in the respective controls.

Inset: Binding of actin-tropomyosin-troponin (actin-TM-TP) to myosin in the presence of F-actin. ●—●: F-actin was added prior to actin-tropomyosin-troponin; ▲—▲: F-actin added after actin-tropomyosin-troponin. The binding assay (10 ml) contained 1.7 mg myosin, 0.7 mg actin-tropomyosin-troponin and the amounts of F-actin indicated at the abscissa.

Binding of F-actin to myosin in the presence of actin-tropomyosin-troponin (low ionic strength and MgATP present)

Fig. 3 demonstrates that actin-tropomyosin-troponin alters the binding of F-actin to myosin. Figs. 3 A and B present two identically performed experiments with different results. In the experiments of Fig. 3 A only little actin could be bound to myosin in the presence of actin-tropomyosin-troponin. In Fig. 3 B the inhibition of binding by actin-tropomyosin-troponin could be overcome by higher amounts of added F-actin. From 5 experiments of the kind shown in Fig. 3 two gave results as shown in Fig. 3 A, two experiments had results as that of Fig. 3 B and in one experiment rising concentrations of F-actin could only partially overcome the inhibition of F-actin binding exerted by actin-tropomyosin-troponin. Obviously both kinds of actin compete for the same binding sites on myosin. That sometimes actin, which is already bound to myosin cannot be displaced, is further illustrated by the inset of Fig. 3. This picture shows an experiment in which F-actin prevented actin-tropomyosin-troponin from being bound to myosin only, when F-actin was added prior to actin-tropomyosin-troponin.

It should be noted, that in those cases in which bound actin-tropomyosin-troponin could not be displaced by F-actin, about 0.45 mg F-actin/mg myosin can maximally be bound in the absence of actin-tropomyosin-troponin, whereas in those cases in which displacement was possible only about 0.20 mg F-actin/mg myosin has been bound. Those F-actin fibrils which are able to displace bound actin-tropomyosin-troponin, seem to have the tendency to become more densily populated with myosin than those actin fibrils which are not able to displace actin-tropomyosin-troponin. Furthermore, in the presence of actin-tropomyosin-troponin, F-actin binding becomes sigmoid rather than hyperbolic.

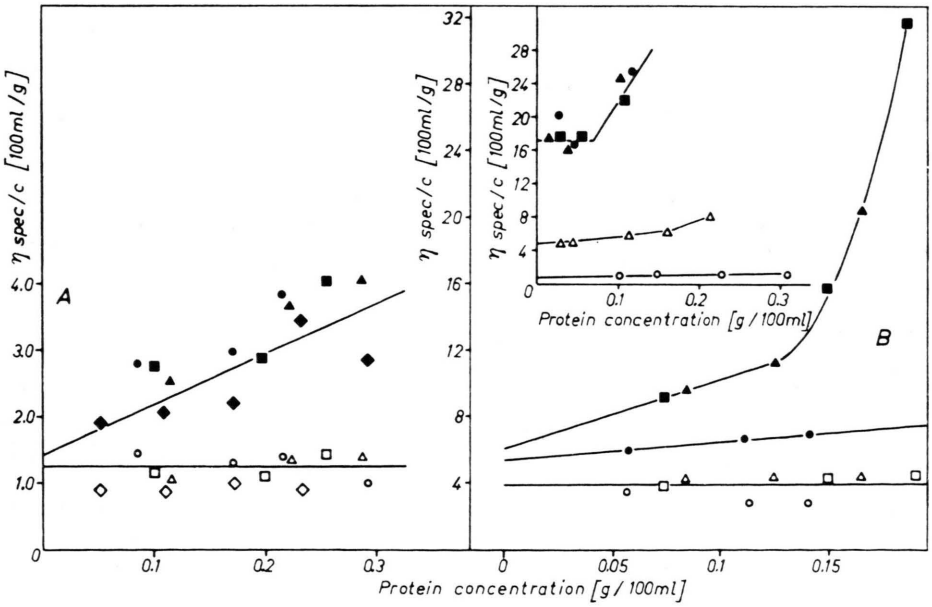

Fig. 4. Reduced viscosity of actin and actomyosin. Actin component either F-actin or actin-tropomyosin-troponin. Conditions: pH 7.2 (tris-maleate buffer 0.02 M) 0.6 M KCl. All protein concentrations expressed in g/100 ml.
A. Viscosity of actomyosin from myosin and actin-tropomyosin-troponin. The following ratios (w/w) of myosin to actin-tropomyosin-troponin are used: 1 : 0.9 (●—●); 1 : 1.16 (■—■); 1 : 1.45 (▲—▲); 1 : 3 (◆—◆); Closed symbols: Without ATP, open symbols: 1 mM ATP and 1 mM $MgCl_2$.

B. Viscosity of actomyosin from F-actin. Myosin : actin (w/w) 1 : 0.2 (●—●); 1 : 0.55 (■—■); 1 : 0.8 (▲—▲). Closed symbols: Without ATP, open symbols: 1 mM ATP, 1 mM $MgCl_2$. Inset: Viscosity of F-actin (upper curve); mixture of 30% F-actin and 70% tropomyosin-troponin (medium curve); and actin-tropomyosin-troponin (lower curve). The tropomyosin-troponin was prepared according to [32] from alcohol-ether dried myofibrils. Different symbols in the F-actin curve refer to different experiments.

Interaction of actin and myosin as revealed by viscosity measurements

The different kind of interaction of myosin with F-actin or actin-tropomyosin-troponin is further illustrated by the viscosity of the respective actomyosins in 0.6 M KCl. In Fig. 4 the reduced viscosities of actomyosins formed from either F-actin or actin-tropomyosin-troponin are compared. When myosin is combined with a small amount of F-actin the reduced viscosity of the resulting actomyosin is linearily related to protein concentration; with larger amounts of F-actin, however, the reduced viscosity increases in an unlinear manner with protein concentration. The reduced viscosity of the actomyosins formed from actin-tropomyosin-troponin increases linearily with protein concentration in the entire concentration range. The actomyosins with the highest content of actin-tropomyosin-troponin contain so much actin as part of actin-tropomyosin-troponin, that one should expect an unlinear concentration dependence if their reduced viscosities would behave like that of F-actin-actomyosins. That this difference in viscosity is due to properties of the respective actins is suggested by the inset of Fig. 4. It can be seen that the reduced viscosity of F-actin is much higher and much more strongly dependent on protein concentration than that of actin-tropomyosin-troponin. That this viscosity difference is not simply due to "dilution" of actin by the regulatory proteins can be inferred from the fact, that the reduced viscosity of a mixture of 30% F-actin and 70% tropomyosin-troponin has still a higher reduced viscosity than that of the actin-tropomyosin-troponin preparation used in this paper. The strong non-linear dependence on protein concentration of actomyosin viscosity is completely abolished by ATP, so that the reduced viscosity, which is now very low, is no longer concentration-dependent.

Interaction of tryptic digested actin-tropomyosin-troponin with myosin.

F-actin is very resistant against tryptic digestion ([13]; own control experiments not shown in detail), but digestion of actin-tropomyosin-troponin reduces the ability of this actin preparation to activate myosin ATPase at low ionic strength considerably. This is shown in Fig. 5. The ATPase activity of actomyosin formed from actin-tropomyosin-troponin declines to about 50% of the initial value after about 30 sec of digestion of actin-tropomysin-troponin. The amount of radioactivity, which is bound to myosin by means of actin-tropomyosin-troponin is even more reduced. It is, however, difficult to say how much protein has been removed by digestion, because we do not know how the radioactive NEM is distributed over the different parts of actin-tropomysin-troponin. One must, however, assume that most of the radioactivity is bound to the tropomyosin-troponin part, because, according to our own experience, F-actin is rarely labelled under the conditions, which are used to combine actin-tropomyosin-troponin with radioactive NEM. The result of Fig. 5 is therefore

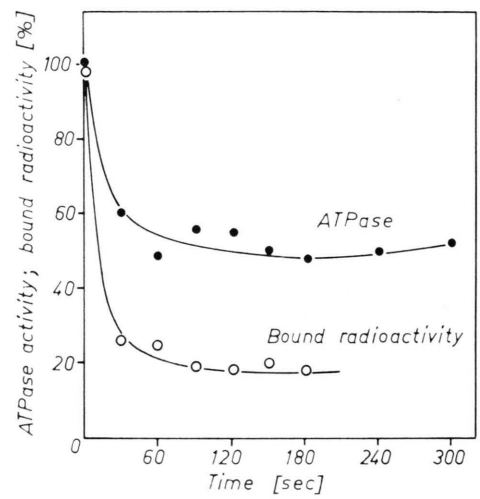

Fig. 5. Reduction by tryptic digestion of the ability of actin-tropomyosin-troponin to activate myosin ATPase. Actin-tropomyosin-troponin was digested for the times indicated at the abscissa and then added to myosin (1.8 mg actin-tropomyosin-troponin to 1.5 mg myosin in 10 ml). 2 mM ATP, 2 mM $MgCl_2$, pH 7.2, 0.03 M KCl. ●—●: ATPase activity; ○—○: Radioactivity which is bound to myosin. 100% refer to the respective values of myosin plus undigested actin-tropomyosin-troponin.

consistent with the view that tryptic digestion affects the tropomyosin-troponin part more than the actin part of actin-tropomyosin-troponin. This is supported by the results of SDS-polyacrylamide gel electrophoresis. Fig. 6 * compares electrophoretograms of undigested and tryptic digested actin-tro-

* Fig. 6 see Table page 408 a.

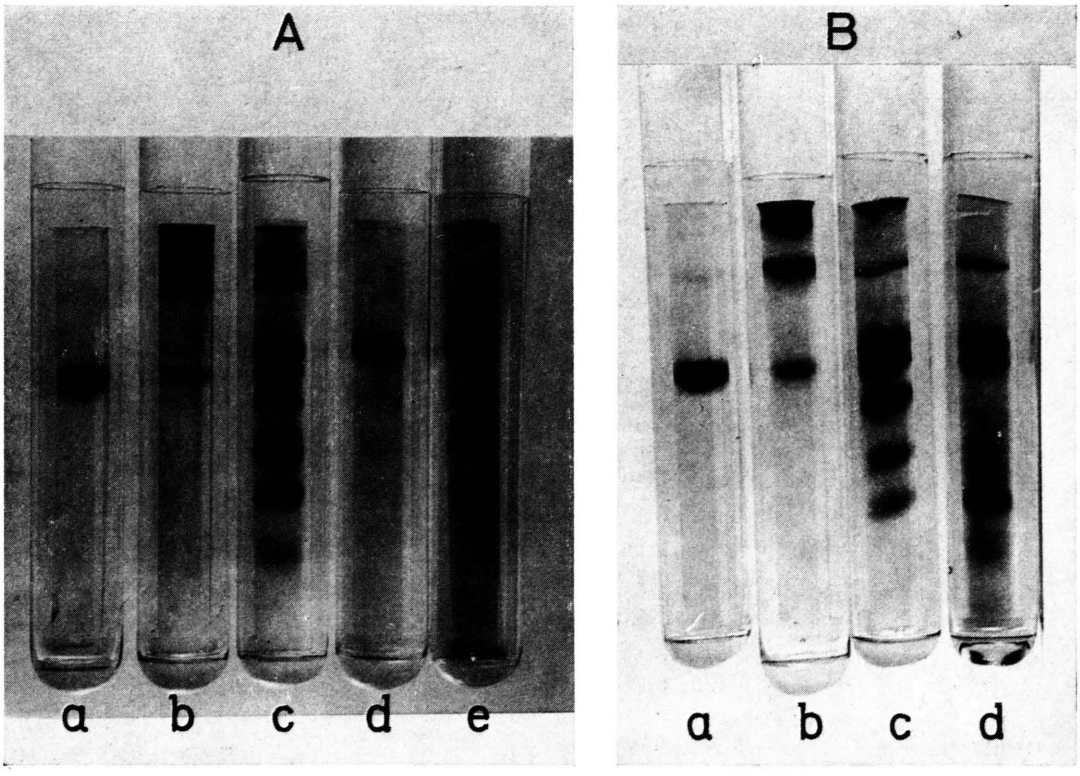

Fig. 6. SDS polyacrylamide gel electrophoresis of different muscle proteins.
A. a: Actin; b: α-Actinin (prepared according to [33], actin band still visible); c: Actin-tropomyosin-troponin; d: Tropomyosin; e: Tropomyosin. (After precipitation of actin-tropomyosin-troponin by ammonium sulfate tropomyosin was precipitated from the remaining supernatant by 60% saturated ammonium sulfate). The following bands can be identified in actin-tropomyosin-troponin (from top to bottom, *i. e.* in the direction of migration): α-Actinin, tropomyosin, actin and four troponin bands, the fourth of which is not in every run clearly demarcated.
B. a: Actin; b: α-Actinin (actin band still visible); c: Actin-tropomyosin-troponin; d: Trypsin-digested actin-tropomyosin-troponin.

pomyosin-troponin with those of other myofibrillar proteins. One can identify α-actinin, tropomyosin, actin and 4 troponin bands (3 main bands and one faint one, which is only seen if one applies sufficient protein to the gel). From the intensities of the different bands one can infer that about one third of the preparation of actin-tropomyosin-troponin consists of actin. The mobility of the three main bands of troponin reflects molecular weights of about 32 000, 25 000, and 20 000 respectively and may thus correspond to the "37 000-" the "23 000-" and "18 000-component" of Wilkinson *et al.* [14]. The last faint band reflects a molecular weight of about 13 000 and may be a proteolytic product [14]. The mobility of the tropomyosin band corresponds to a molecular weight of about 60 000, which is nearly the molecular weight of the tropomyosin dimer which is 70 000 [15]. This indicates that tropomyosin migrates under our conditions (proteins were not or only shortly incubated with mercaptoethanol at room temperature) as a dimer.

From Fig. 6 B it can be seen that after one minute of digestion the first and second troponin bands have disappeared, the third one, however, remains and becomes even stronger. This third band is analogous to the "18 000-component" of Wilkinson *et al.* [14] and should therefore correspond to the "calcium-sensitizing factor" of Schaub *et al.* [16] and Wilkinson *et al.* [14] or to troponin A (Hartshorne and Pyuyn [17]). Obviously, the abolition of Ca-sensitivity of actomyosin ATPase after tryptic digestion of troponin is not due to inactivation of the "calcium-sensitizing factor" of troponin. These results agree with recent findings of Drabikowski *et al.* [18].

If this reduced activating effect of actin-tropomyosin-troponin on myosin ATPase at low ionic strength and in the presence of Mg should be due to inactivation of actin by trypsin this effect of trypsin must have been so small that it could not be detected by SDS gel electrophoresis. Since the disappearance of the first and second troponin band is the most conspicuous result of trypsin digestion, one can suppose that actin-tropomyosin-troponin activates myosin ATPase to its full extent only if the troponin system is unimpaired. It has already been shown by Dancker [1] that the ATPase of actomyosin which contains the regulatory proteins is reduced after tryptic digestion of these proteins.

Discussion

Binding in 0.25 M KCl in the absence of ATP

For the interpretation of the binding experiments the following points have to be taken into consideration. It is the radioactive myosin or actin in the pellet, that is considered as "bound". Free actin is soluble; in the experiments performed in 0.25 M KCl free myosin is likewise soluble. Hence actin will be found in the pellet only as part of actomyosin. A double-stranded F-actin polymer may have an average length of about 5000 Å (Kawamura and Maryama [19]) and may possess about 300 actin monomers. If one myosin molecule binds with its two S_1-fragments (*cf.* Lowey *et al.* [20]) two actin monomers, 1 mg of myosin would bind 0.18 mg actin (molecular weight of myosin and actin 500 000 and 45 000 dalton, respectively). This, however, is only the upper limit. If only 10% of the actin units of the F-actin fibrils are bound to myosin one has to expect 1.8 mg actin per mg of myosin. But an F-actin fibril having bound only little myosin may behave like free actin and may remain in the supernatant. Increasing amounts of actin added to myosin may result in very actin-rich actomyosins which finally remain soluble and are consequently no longer found in the pellet. As a result, rather complex binding curves can be expected and are really found (compare Fig. 1). In the absence of ATP the proportion of actin to myosin in actomyosin depends strongly on the proportion of the total amount of the added proteins. Myosin seems to be distributed more or less statistically over all actin fibrils present in the system. The more actin fibrils are offered, the less saturated is each single fibril. In the absence of ATP there is no preferential binding of myosin to those actin fibrils which have already bound other myosin molecules. Provided, enough myosin is offered to a given amount of actin fibrils, the actin-myosin ratio approaches a value which indicates that every two actins are combined with one myosin molecule or that each myosin molecule binds two actins. Accordingly, the molar ratio of myosin to actin is 1 : 2. This has already been inferred from the experiments of Gergely and Kohler [21] and Tonomura *et al.* [22], although the electromicrographs of Huxley [23] as well as the ultracentrifugation studies of Young [24] suggest for HMM a molar binding ratio of 1 : 1. On the other hand recent ultra-

centrifugation studies of Eisenberg *et al.* [25] and Margossian and Lowey [26] indicate that 1 H-meromyosin molecule binds two actin subunits.

Binding of actin to myosin in the presence of ATP and low ionic strength

The following three main results have emerged from the studies of actin-myosin interaction in the presence of MgATP and low ionic strength, that is under conditions under which the specific energy-yielding and ATP-consuming interaction between actin and myosin takes place.

1. In the presence of MgATP less actin is bound to myosin than in the absence of ATP.

2. When increasing amounts of actin are added to a constant amount of myosin the total amount of myosin already appears in the pellet when the amount of actin in the pellet still increases. Activation of ATPase by actin is described by a curve that corresponds to the appearance of actin rather than of myosin in the pellet.

3. As far as half saturation of actin binding can be inferred from the binding in the absence of ATP, one can deduce that the affinity of actin to myosin has not significantly decreased when MgATP has been added.

To point 1: The results may be explained by assuming that in the presence of MgATP the F-actin fibrils have lost part of their actin units, which are now no longer able to bind to myosin. Indeed, Kawamura and Maruyama [19] have shown that the average particle length of actomyosin decreases in the presence of ATP. From their electronmicrographs, however, it cannot be decided if this decrease in average particle length is due to a loss of material. One mechanism by which loss of actin could have happened, would have been partial depolymerisation of the existing actin fibrils. Depolymerisation of F-actin by ATP requires, however, an even lower ionic strength and more time than is available in the experiments described in this paper. Furthermore, light scattering and viscosity measurements (not outlined in detail) failed to detect depolymerisation under the present conditions. Our results are consistent with the view that in the presence of MgATP a rearrangement between actin and myosin must have taken place with the result that myosin now binds preferentially to fewer actin fibrils leaving other actin fibrils free. Such a rearrangement can further be inferred from the finding that only those actins which become densily occupied with myosin are able to displace bound actin-tropomyosin-troponin (Fig. 3). It looks as if in the presence of MgATP there exist F-actin fibrils which have a strong tendency to become fully saturated with myosin at the cost of other actins. These considerations imply that a population of F-actin fibrils, which is homogeneous with respect to its binding behaviour towards myosin in the absence of ATP becomes heterogeneous in the presence of MgATP (but not inorganic pyrophosphate). Only a certain amount of actin fibrils present interacts in the presence of MgATP with myosin. This may result from either of two possibilities. Either those fibrils which do bind myosin have increased their affinity towards myosin, so that they become more strongly saturated at the cost of the other fibrils with lower affinity, or those fibrils which do not bind myosin any more have completely lost their affinity towards myosin in the presence of MgATP so that the myosin must necessarily be more densily distributed over the rest of the fibrils.

To point 2: Myosin in the absence of actin is completely soluble in the presence of low ionic strength and MgATP (Table I). On the other hand, it is completely precipitated when only a part of it has bound actin (Fig. 2, Table I). Obviously, the binding of few myosin molecules to actin promotes aggregation to myosin filaments of all the myosin molecules present. These newly formed filaments possess still free binding sites for actin and have accordingly not yet the full ATPase activity. That actin promotes the forming of myosin filaments can further be inferred from the long known experience (which is used in experiments like that of Fig. 1 A) that in the absence of ATP actomyosin precipitates at a higher ionic strength than myosin alone. In the presence of MgATP the first monomeric myosin molecules which subsequently give rise to filament formation must have been bound to actin with a presumably very high affinity which seems to be much higher than that of HMM or HMM-S_1, which both are monomeric too but which lack the long "tail" of the myosin molecule. If so, one has to assume that actin-myosin interaction is deeply influenced by the LMM-part, the tail, of the myosin molecule.

To point 3: That actin-myosin affinity is only little affected by MgATP under the present condi-

tions, is strongly indicated by the presented results and further supported by the observation that inorganic pyrophosphate has only a small effect on actin-myosin binding. This is in marked contrast to HMM, the apparent affinity of which to actin is decreased by ATP about 200fold (Eisenberg *et al.* [25], *cf.* Margossian and Lowey [26]). Quite obviously, the actin-myosin complex in the presence of MgATP, that is under conditions under which the contractile ATPase is active, is a steady-state complex with a certain flow of actin through it. One can write a simple kinetic scheme (*cf.* also Szentkiralyi and Oplatka [12]) which allows only very roughly for the refinements which the interpretation of myosin ATPase has experienced in recent years (*cf.* Lymn and Taylor [27]).

$$A + M\text{-}ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$$

$$A\text{-}M\text{-}ADP + P_i \xrightarrow{k_2\,(ATP)} A + M\text{-}ATP + ADP\ ;$$

(A = actin, M = myosin).

The presented results are consistent with the assumption, that the ratio k_1/k_{-1}, the equilibrium constant of actin-myosin binding, is under the present conditions independent of ATP. If so, one must further assume, that in myosin $k_{-1} \gg k_2$, so that half-saturation in the presence of MgATP reflects the true equilibrium constant, whereas in HMM $k_2 \cong k_{-1}$, so that a higher half-saturation concentration of actin would prevail in the presence of MgATP. That k_2 is at least larger in HMM than in myosin can be deduced from the higher maximal ATPase activity of HMM as compared to myosin (Eisenberg and Moos [11]) which should equal $k_2 \cdot \text{myosin}_{\text{total}}$.

The following point may be mentioned in this context. With HMM it is difficult to detect physical interaction between actin and myosin, when enzymatic interaction takes place (Leadbeater and Perry [28], Eisenberg and Moos [29], Eisenberg *et al.* [30]) whereas, as we have seen, in intact myosin the enzymatically active steady state complex cannot only be inferred from ATPase measurements but can easily be isolated. The concentration of this enzymatically active complex depends, as we have further seen, on the concentration of free actin and free myosin. Accordingly, in order to fully saturate myosin with actin it is necessary to add more than the stoichiometric amount of actin.

Interaction of myosin with actin-tropomyosin-troponin

The preceding discussion has considered how the form of myosin may influence actin-myosin interaction. That, on the other hand, the state of actin modifies actin-myosin interaction can be deduced from the experiments in which actin-tropomysin-troponin is present. It looks as if myosin behaves differently towards F-actin when actin-tropomyosin-troponin, which is already bound, has to be displaced (Fig. 3), since F-actin displays now a sigmoid rather than a hyperbolic binding curve. The actin-tropomyosin-troponin preparation does not only differ from F-actin in that it contains the regulatory proteins but also in the properties of its actin part. Actin-tropomyosin-troponin is always obtained from actin extracts when F-actin has been precipitated by ultracentrifugation. Obviously, the regulatory proteins which remain in the supernatant can bind small amounts of actin, which seem to be less polymerized than the F-actin already removed. This interpretation is supported by the viscosity measurements. The low actin content of actin-tropomyosin-troponin distinguishes it from actin-tropomyosin-troponin complexes described in the literature [6]. F-actin and tropomyosin-troponin form complexes with about twice as much actin (by weight) as tropomyosin-troponin (Spudich *et al.* [7], Spudich and Watt [6], Maruyama and Ebashi [31]). Accordingly, it seems that our preparation is more an actin-containing preparation of tropomyosin-troponin; but on the other hand, if there would be an excess of free tropomyosin-troponin one should expect, that, when radioactive actin-tropomyosin-troponin is displaced by F-actin (Fig. 3) most of the radioactivity which belongs most likely more to the tropomyosin-troponin part than to the actin part of actin-tropomyosin-troponin should now move to F-actin and hence should remain bound. This, however, is not the case so that one has to assume that the whole actin-tropomyosin-troponin and not only its actin part is displaced from myosin. This actin-tropomyosin-troponin preparation must therefore be more than a mere mixture of actin and tropomyosin-troponin.

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