The Modification of Actomyosin ATPase Activity by Tropomyosin-Troponin and its Dependence on Ionic Strength, ATP-Concentration, and Actin-Myosin Ratio

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Actomyosin ATPase, Tropomyosin-Troponin, Ionic Strength

At millimolar concentrations of ATP the ATPase activity of regulated actomyosin (which consisted of myosin and of actin containing the regulatory proteins tropomyosin and troponin) was lower than that of unregulated actomyosin (containing actin devoid of the regulatory proteins) when the ionic strength was high (>0.03 M KCl). At low ionic strength (0.03 M KCl) the ATPase activity of regulated actomyosin was similar to or even higher than that of unregulated actomyosin. Besides increasing ionic strength an increasing actin-myosin ratio tended to depress the ATPase activity of regulated actomyosin below that of unregulated one. At lower ATP concentrations (0.1 mM or lower) the ATPase activity of regulated actomyosin was higher than that of unregulated actomyosin at any ionic strength and at any actin-myosin ratio. EGTA inhibited the ATPase of regulated actomyosin under any conditions at high ATP concentrations. At lower ATP concentrations EGTA inhibited either at higher ionic strength or at a higher actin-myosin ratio. The inhibition of the ATPase activity of acto-HMM by increasing ionic strength was not influenced by the regulatory proteins. — For the interpretation of these results it has been assumed that in actomyosin regulated actin can adopt three states: A low-affinity state which activates the ATPase of myosin only slightly (occurring at high ATP concentrations and in the absence of Ca^{2+}), a high affinity state which activates the ATPase of myosin better than does unregulated actin (occurring at low concentrations of ATP and in the presence of Ca^{2+}), and an intermediate state. This latter state (occurring at high concentrations of ATP and in the presence of Ca^{2+} or at low concentrations of ATP and in the absence of Ca^{2+}) activates the ATPase of myosin less than does unregulated actin when the actin-myosin ratio is high (wide spacing of myosin on the actin filaments) but activates more (or at least not less) when the actin-myosin ratio is low (dense spacing of myosin on the actin filaments).

Introduction

The Mg-activated ATPase activity of actomyosin requires for its optimal function a restricted range of low ionic strength (Hasselbach [1], Dancker and Hasselbach [2]). Likewise, tension development of muscle fiber preparations is optimal only at a low range of ionic strength (Gordon *et al.* [3]). According to Eisenberg and Moos [4], at high ionic strength a higher concentration of actin for half-maximal activation of HMM-ATPase is required than at low ionic strength. Obviously, increasing ionic strength favors the dissociation of actin from myosin by ATP thus leading to a state corresponding to relaxation in muscle.

In muscle, however, the ionic milieu does not change during activity and rest to such an extent that would be necessary to explain activation and desactivation of muscular activity. One therefore has to assume that properties of the contractile proteins themselves have to be altered in such a way that the existing conditions favor at one time interaction of actin and myosin and at another time dissociation of actin and myosin. It is now a generally accepted view that the regulatory proteins tropomyosin and troponin which are part of the thin filaments of muscle respond in some way to the intracellular concentration of Ca ions thus determining whether the prevailing conditions favor actin-myosin interaction or actin-myosin dissocia-

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Abbreviations: EGTA, ethyleneglycol-2-(2-aminoethyl)-tetra-acetic acid; HMM, heavy meromyosin; S-1, subfragment 1 of HMM; PEP, phosphoenol pyruvate; PK, piruvate kinase.

Enzymes: ATPase (ATP phosphohydrolase), EC 3.6.1.3; pyruvate kinase (ATP: pyruvate 2-O-phosphotransferase), EC 2.7.1.40.

tion (for reviews see Ebashi and Endo [5], Weber and Murray [6]).

Because actin free from regulatory proteins activates myosin ATPase also in the absence of Ca ions it has originally been concluded that pure actin at any Ca concentration is equivalent to actin-tropomyosin-troponin in the presence of Ca^{2+}. In recent years, however, evidence has accumulated which indicates that the regulatory proteins not only confer Ca-sensitivity on actin but alter other properties of actin too [7–12].

The present paper investigates to what extent myosin ATPase activated by regulated actin (a term used by Bremel *et al.* [11] denoting actin that contains the regulatory proteins) differs from that activated by unregulated actin in its response towards ionic strength and varying concentrations of ATP.

Methods

Actin and myosin were prepared as described earlier [12]. Tropomyosin-troponin has sometimes been prepared following the procedure of Hartshorne and Mueller [13] (extraction of alcohol-ether dried myofibrils with 1 M KCl). In most cases, however, a procedure has been applied which combines the extraction method described by Hitchcock [14] with the experience of Hartshorne and Dreizen [15] that acidic pH inhibitis proteolytic activity associated with troponin preparations. After extraction of myosin from minced rabbit muscle by 0.6 M KCl the muscle residue was washed free from salt with de-ionized water, acidified to pH 1.5 – 2.0 by HCl and extracted overnight (about 1.2 l extract for the muscle residue obtained from one rabbit). This extract was then neutralized by KOH and separated from the residue by centrifugation. The protein fraction obtained between 40% and 60% saturation with $(NH_4)_2SO_4$ (to 100 ml solution 24.3 g and then additional 12.15 g ammonium sulfate were added at pH 7.2) was collected, dissolved in water and dialyzed against 0.05 M KCl, 0.01 M tris-maleate buffer, pH 7.2, and subsequently clarified by 1 hour centrifugation at 50 000 × *g*. All procedures have been done in the cold. This preparation which possessed good tropomyosin-troponin activity was stored until used at −17 °C. For obtaining "regulated actin" (a term introduced by A. Weber and co-workers [10, 11]) this preparation was mixed with F-actin if not otherwise stated in a 1:1 ratio (w/w) prior to the addition to the splitting assay. Consequently, "regulated actomyosin" means an actomyosin composed of myosin and regulated actin. "Unregulated actin" refers to actin free from the regulatory proteins.

HMM was prepared according to Eisenberg and Moos [4], using 1/250 (w/w) trypsin for 10 min.

ATPase measurements were done as described earlier [12]. When the ATP concentration was below 1 mM the assay contained 1.5 mM phosphoenol pyruvate and 0.04 mg/ml pyruvate kinase. PEP and PK were also included into assays containing higher concentrations of ATP when the action of different ATP concentrations should be compared. The stock solution of PK was suspended in 3.2 M ammonium sulfate, hence the ATPase assays containing PK contained 0.012 M $(NH_4)_2SO_4$.

When the amount of myosin was the same in every ATPase assay of one experiment the ATPase activity indicated at the ordinate was proportional to the ATPase activity per mg myosin. When, however, as in Fig. 3 B and C and in Fig. 5 the ATPase activities of different amounts of myosin (actin remaining constant) were compared, the ATPase activity was not divided by the respective amounts of myosin but represented the total ATPase activity of the different assays. Consequently the values of ATPase activity of these experiments were related to the degree of saturation of actin with myosin.

The actin concentrations indicated in the figures and legends refer always to the actin part of the respective preparations.

ATP was obtained from Waldhof (Mannheim, Germany), EGTA from Fluka (Buchs, Switzerland). Trypsin and soybean trypsin inhibitor were purchased from Serva (Heidelberg, Germany) and PEP and PK from Boehringer (Mannheim, Germany).

Results

The dependence of ATPase activity of actomyosin on ionic strength at high concentrations of ATP

At millimolar concentrations of ATP the regulatory proteins altered the dependence of actomyosin ATPase activity on ionic strength in either of two ways. For both ways examples are given in Fig. 1 A and B. Sometimes the ATPase activity of regulated actomyosin was higher at low ionic strength but lower at higher ionic strength than that of unregulated actomyosin, in other experiments the ATPase activity of regulated actomyosin was lower at any ionic strength (Fig. 1 B). In both cases, however, the ATPase activity of regulated actomyosin declined more steeply with increasing ionic strength than that of unregulated actomyosin.

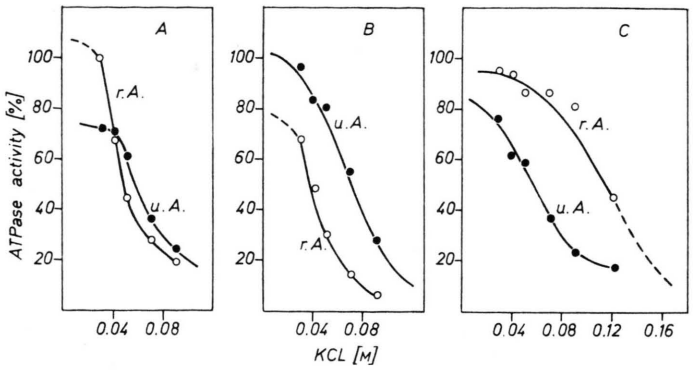

Fig. 1. ATPase activity of unregulated and regulated actomyosin at different values of ionic strength. A and B: 2 mM ATP and 2 mM $MgCl_2$, 0.22 mg/ml myosin, 0.01 mg/ml actin (A) or 0.1 mg/ml actin (B). C: 0.1 mM ATP, 0.1 mM $MgCl_2$ (PEP and PK present), 0.16 mg/ml myosin, 0.045 mg/ml actin. 100% ATPase activity (μmol Pi × mg myosin^{-1} × min^{-1}): A 0.3, B and C 0.2. Symbols: ● unregulated actin (u. A.); ○ regulated actin (r. A.).

The dependence of actomyosin ATPase activity on ionic strength at low concentrations of ATP

At lower concentrations of ATP (0.1 mM) the ATPase activity of regulated actomyosin was at every ionic strength higher than that of unregulated actomyosin (see Fig. 4 A). Obviously, the ATPase activity of regulated actomyosin is at low concentrations of ATP not as sensitive against increasing ionic strength as that of unregulated actomyosin. This interpretation is supported by the experiment which is depicted in Fig. 1 C. In this experiment the higher ATPase activity of regulated actomyosin can be described as a shift of the activity curve to higher values of ionic strength.

The importance of the actin-myosin ratio for the behavior of regulated actomyosin

The results described so far show that at lower concentrations of ATP regulated actin activated the ATPase activity more efficiently than did unregulated actin whereas at high concentrations of ATP the ATPase activity of regulated actomyosin was mostly lower than that of unregulated actomyosin although in some cases (*cf.* Fig. 1 A) the ATPase activity was at low values of ionic strength higher than that of unregulated actomyosin. In search of an explanation for this difference (compare Fig. 1 A and Fig. 1 B) we investigated the role of actin-myosin ratio, because it was shown by Bremel and Weber [10], Bremel *et al.* [11], Weber and Murray [6] that this ratio might influence the ATPase activity of acto-HMM or acto-S-1. The experiments of Fig. 1 A and 1 B differ only in the actin concentration of the splitting assay. From these two experiments one has to conclude that the tendency of regulated actomyosin to have a lower ATPase activity than unregulated actomyosin is more pronounced at higher concentrations of regulated actin. This assumption is supported by experiments described in Fig. 2 B. When, at high concentrations of ATP and in the presence of Ca ions, varying amounts of unregulated actin or of regulated actin were added to myosin, regulated actin activated myosin ATPase at lower actin concentrations to a higher extent, at higher actin concentrations, however, to a smaller extent than did unregulated actin. This effect depended not only on the concentration of total actin but de-

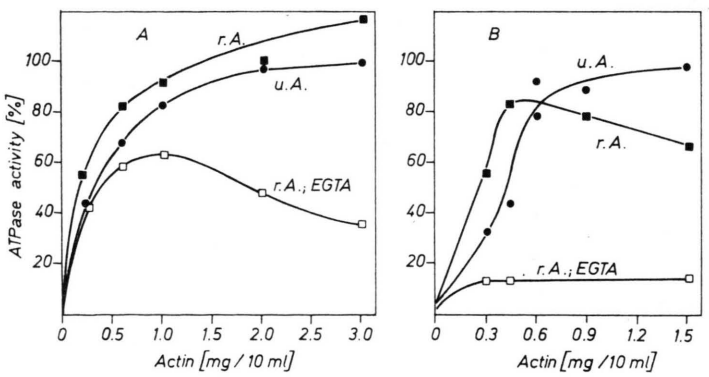

Fig. 2. The dependence of actomyosin ATPase activity on the concentration of actin at different concentrations of ATP. A: 0.1 mM ATP and $MgCl_2$ (PEP and PK present), B: 2 mM ATP and $MgCl_2$, 0.18 mg/ml myosin. 100% ATPase activity (μmol Pi × mg myosin^{-1} × min^{-1}): A 0.24, B 0.38. Symbols: ● unregulated actin (u. A.); ■ regulated actin (r. A.); □ regulated actin 1 mM EGTA. — The sigmoidal form of the actin curve in Fig. 2 B did not occur in every experiment; its significance is unknown.

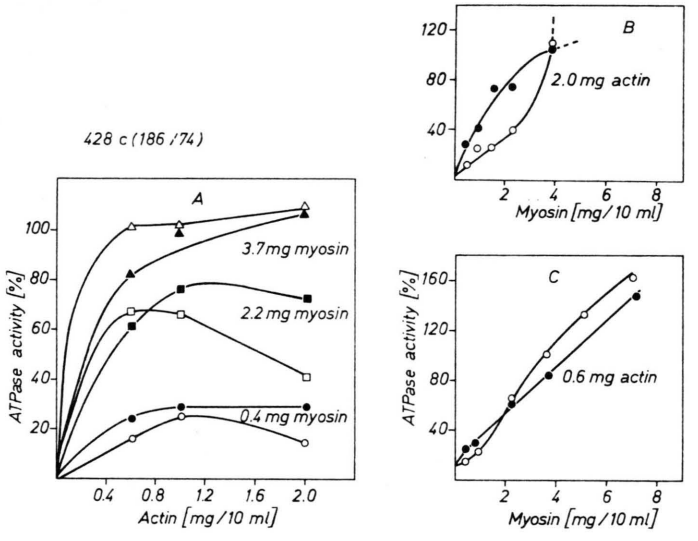

Fig. 3. ATPase activity of unregulated and regulated actomyosin at different ratios of actin to myosin. 2 mM ATP and $MgCl_2$. 100% ATPase activity (μmol Pi × ml assay^{-1} × min^{-1}) : 0.06. A: ATPase activity as a function of actin concentration at different myosin concentrations in the 10 ml assay. B and C: ATPase activity as a function of myosin concentration at 2 mg/10 ml actin (B) or 0.6 mg/ml actin (C). Open symbols: Regulated actin, full symbols: Unregulated actin.

pended on the concentration of myosin too. As can be seen from Fig. 3 A, the activation by regulated actin was especially superior to unregulated actin when the myosin concentration in the splitting assay was high. At moderate myosin concentrations, the change from higher to lower ATPase activity of regulated actomyosin (as compared to unregulated one) occurred at a lower actin concentration, and at the lowest myosin concentration the ATPase activity of regulated actomyosin was always lower than that of unregulated actomyosin. This behaviour is further illustrated by comparing the ATPase activity of regulated and unregulated actomyosin which differed in their myosin content. Figs 3 B and 3 C compare the ATPase activity of splitting assays in which the concentration of myosin was different whereas that of actin remained constant. As can be seen, the ATPase activity of regulated actomyosin was lower than that of unregulated one when the myosin concentration was low (particularly when actin was present at a high concentration as in Fig. 3 B), but was higher at higher myosin concentrations (particularly when actin was present at a lower concentration as in Fig. 3 C). Obviously, at l o w actin-myosin ratios regulated actin activated myosin ATPase more than did unregulated actin, but at h i g h e r actin-myosin ratios regulated actin activated less. From the experiments demonstrated in Figs 2 and 3 it can be deduced that in the case of regulated actin the increase of actin-activated ATPase activity with increasing myosin concentrations cannot be described by a hyperbolic adsorption isotherm. Instead, one has to assume that the activation of myosin ATPase by regulated actin was under the described conditions a cooperative phenomenon. When at low concentrations of free myosin the saturation of actin with myosin was low and the spacing of myosin on the actin filament was wide myosin ATPase was less activated by regulated actin than when at high concentrations of myosin the saturation of actin was high and the spacing of myosin on actin was dense.

The ATPase inhibition by EGTA and its dependence on ATP concentration, ionic strength, and actin-myosin ratio

At high concentrations of ATP the ATPase activity of regulated actomyosin was maximally inhibited by Ca removal already at 0.03 M KCl and at all concentrations of regulated actin. At lower concentrations of ATP, however, EGTA inhibition required some additional conditions. In Fig. 4 A an experiment is shown in which the inhibition by EGTA at 0.1 mM ATP increased with increasing ionic strength. Besides higher values of ionic strength higher concentrations of regulated actin favored the inhibition by EGTA at low concentrations of ATP: The experiment of Fig. 2 A shows that already at low ionic strength (0.03 M KCl) inhibition by EGTA could be seen when the concentration of regulated actin increased beyond a specific value.

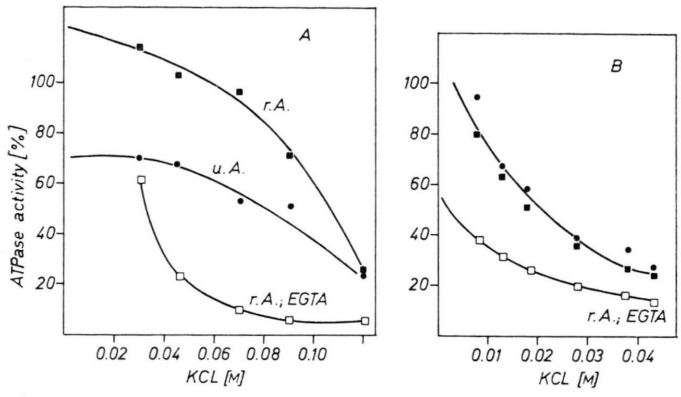

Fig. 4. ATPase activity of actomyosin and acto-H-meromyosin at different values of ionic strength. A: Actomyosin (0.22 mg/ml myosin, 0.06 mg/ml actin), 0.1 mM ATP and $MgCl_2$ (PEP and PK present). B: Acto-HMM (0.185 mg/ml HMM, 0.5 mg/ml actin), 2 mM ATP and $MgCl_2$ (no PEP and PK present). 100% ATPase activity (μmol Pi × mg myosin^{-1} × min^{-1}): A 0.2, B 0.22. Symbols: ● unregulated actin (u. A.); ■ regulated actin (r. A.); □ regulated actin, 1 mM EGTA.

The dependence of EGTA inhibition on the actin-myosin ratio at lower ATP concentrations could be demonstrated in quite the same way at is was done with the interaction between myosin and regulated actin at higher concentrations of ATP (Figs 3 B and C). From Fig. 5 one can see that at 0.1 mM

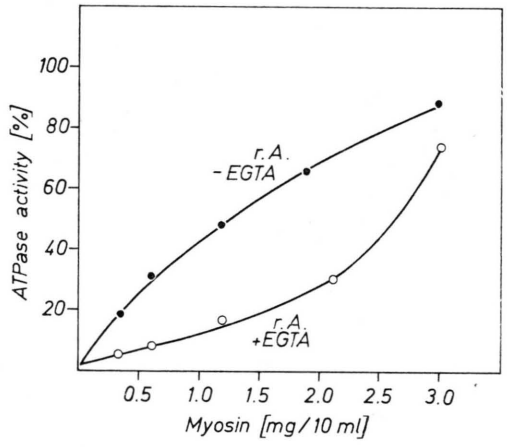

Fig. 5. Fig. 5. EGTA inhibition of actomyosin ATPase at 0.1 mM ATP and its dependence on the ratio of actin to myosin. The ATPase activity of regulated actomyosin which differed in its myosin content was measured in the absence and presence of 1 mM EGTA. 0.1 mM ATP and MgC_2 (PEP and PK present). The assays (10 ml) contained 0.54 mg actin and 1.0 mg tropomyosin-troponin and the amounts of myosin indicated at the abscissa. 100% ATPase activity (μmol Pi × ml assay^{-1} × min^{-1}) 0.09. Symbols: ● no EGTA; ○ 1 mM EGTA.

ATP the EGTA inhibition disappeared with increasing myosin concentrations (equivalent to a decreasing actin-myosin ratio). However, the ATPase activity of regulated actomyosin was at 0.1 mM ATP and in the presence of 0.1 mM EGTA never higher than in the absence of EGTA. Obviously, the point has not been reached at which the sigmoidal curve (describing the interaction in the presence of EGTA) intersects the hyperbolic curve (describing the interaction in the absence of EGTA).

It is known since long that inhibition of actomyosin ATPase by Ca-removal requires besides the regulatory proteins a minimum concentration of nucleoside triphosphate[16]. From the experiments shown in Figs 2 A and 5 it can be inferred that the

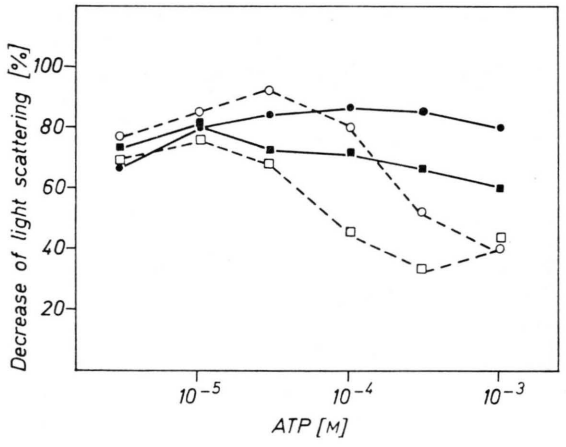

Fig. 6. Superprecipitation of actomyosin containing two different amounts of regulated actin. 0.76 mg/ml myosin. Symbols: ●, ○ 0.013 mg/ml regulated actin; ■, □ 0.13 mg/ml regulated actin; open symbols: measurements in the presence of 1 mM EGTA. — The forming of superprecipitating actomyosin floccules decreased the light scattering (measured with a Hitachi-Perkin-Elmer Fluorescence Spectrophotometer MPE-2A at 400 nm) because the uniformly distributed actomyosin (which strongly scattered) "condensed" after the addition of 1 mM ATP (in the presence of 1 mM $MgCl_2$) to dense particles leaving most of the medium optically empty. The figure shows the extent of this decrease in light scattering due to superprecipitation. High values correspond to a high extent of superprecipitation, low values correspond to a low extent. The inhibition by EGTA started at a lower ATP concentration when the amount of regulated actin was high. — 0.03 M KCl throughout.

concentration of ATP which is necessary for EGTA inhibition depends on the actin-myosin ratio. That this is really the case is demonstrated by an experiment in which the superprecipitation of regulated actomyosin in the absence and presence of EGTA has been measured at different ATP concentrations (Fig. 6). The inhibition of contractile interaction (represented as a reduced decrease of light scattering) by EGTA occurred at a lower ATP concentration when the amount of regulated actin added was high.

The ATPase activity of acto-H-meromyosin at different values of ionic strength and in the presence of the regulatory proteins

The experiments described so far have been done with the entire myosin molecule. Since myosin is able to form filamenteous aggregates under the conditions under which measurements of actomyosin ATPase are done and since it is conceivable that this ability to form filaments may influence the actin-myosin interaction we compared the ionic strength dependence of the ATPase activity of acto-HMM with that of actomyosin, because HMM cannot form filaments.

Fig. 4 B shows the ATPase activity of acto-HMM at different concentrations of KCl and at 2 mM ATP. The ATPase activity of acto-HMM was already low at values of ionic strength at which actomyosin ATPase was still highly active. This may reflect the lower apparent affinity of actin towards HMM in the presence of ATP (Eisenberg and Moos [4]). This low affinity seems to be related to the observation that, as far as measured by means of viscosity (Szent-Györgyi [17], Leadbeater and Perry [18], Eisenberg and Moos [19]) or light scattering (own unpublished observations) ATP appears to dissociate HMM and actin at any ionic strength [20].

The decline of acto-HMM ATPase activity with increasing ionic strength was with regulated actin not steeper than with unregulated actin contrary to the case in actomyosin (compare Fig. 4 B and Fig. 1 A and B). Furthermore the ATPase activity of acto-HMM fell with increasing ionic strength in a manner which resembles a hyperbolic function (convex to the abscissa) whereas the curves referring to the ATPase activity of actomyosin (rather than acto-HMM) were S-shaped or at least concave to the abscissa.

Discussion

This paper describes the ATPase activity of regulated actomyosin (whose actin part contained the regulatory proteins tropomyosin and troponin) and of unregulated actomyosin (whose actin part was devoid of the regulatory proteins) at different values of ionic strength, at different concentrations of ATP, and at different concentrations of actin and myosin.

Influence of tropomyosin-troponin on the ionic strenght dependence of actomyosin ATPase activity

Quite generally it holds that at low ionic strength the ATPase activity of actomyosin is high, but when the ionic strength increases beyond a specific value, the ATPase activity declines drastically (Hasselbach [1]) in a manner which resembles highly cooperative phase transition. This decline occurs at low concentrations of ATP at higher values of ionic strength than at higher concentrations of ATP, hence ATP and ionic strength act in a concerted way. Tropomyosin-troponin, in the presence of Ca^{2+}, modified this general picture in either of two ways: At low concentrations of ATP (about 0.1 mM), actomyosin ATPase activity was at all values of ionic strength higher in the presence than in the absence of tropomyosin-troponin (stabilizing effect of tropomyosin-troponin). At higher concentrations of ATP, however, the transition from activity of ATPase to inactivity occurred within a narrower range of ionic strength in the presence of tropomyosin-troponin. The cooperativity of this transition appeared to be increased by tropomyosin-troponin.

The relationship between ATP concentration and ionic strength

Since dissociation into actin and myosin occurs only when ATP combines with myosin a minimum concentration of ATP is required for any lowering of ATPase activity which is caused by dissociation of actin from myosin. The fact that at low concentrations of ATP ATPase is still active at such values of ionic strength at which it is already inactive at high concentrations of ATP implies that at higher ionic strength, *e. g.* 0.05 M KCl, increasing concentrations of ATP inhibit actomyosin ATPase. Obviously, higher values of ionic strength provide conditions under which actomyosin ATPase can be

fully active only as long as myosin is insufficiently saturated with ATP (saturation of myosin in actomyosin occurs between 0.1 mM and 1 mM ATP[21, 22]). The importance of the degree of saturation of myosin with ATP has been recognized by Bremel *et al.*[11] in order to explain their observation that at lower ATP concentrations the ATPase activity of regulated acto-HMM (or acto-S-1) was higher than that of the unregulated species, but that with increasing ATP concentrations the activity of the regulated acto-HMM (or acto-S-1) decreased to the value of the unregulated species. However, a decline of ATPase activity with increasing concentrations of ATP does not necessarily require regulated actin, it can be seen, as has just been discussed, with unregulated actin as well. It is difficult to say why myosin, uncompletely saturated with ATP behaves differently from fully saturated one. In the case of myosin (rather than HMM) a simple way of thinking is to assume that, if in a myosin filament rigor links (links between actin and myosin in the absence of ATP) still exist, the whole set of myosin molecules of this filament is kept in the vicinity of the adjacent actin filament thus counteracting dissociation.

The difference in ATPase activity between regulated and unregulated actomyosin

Table I summarizes the conditions under which the ATPase activities of regulated and of unregulated actomyosin have been found to be different. It can be seen that, depending on the conditions, regulated actin can be superior as well as inferior to unregulated actin in its ability to activate the ATPase of myosin. The table shows that high concentrations of ATP, high values of ionic strength, Ca-removal, and high actin-myosin ratio tended to depress the ATPase activity of regulated actomyosin. However, none of these conditions alone was sufficient to lower the ATPase activity of regulated actomyosin below that of unregulated actomyosin. At least two of the conditions had to be fulfilled. Hence, at high concentration of ATP (which by itself was a "inhibitory" condition) only one additional condition had to be present whereas at low concentrations of ATP two factors had to be combined. However, not every combination of conditions was possible. At low concentration of ATP the combination of high ionic strength and high concentration of regulated actin was not found to lower the ATPase activity of regulated actomyosin with respect to unregulated one. Instead, the ATPase activity of regulated actomyosin was at low concentrations of ATP more resistant against ionic strength at any actin-myosin ratio.

The conditions under which regulated actomyosin had a higher ATPase than unregulated one were just opposite to those which inhibited regulated actomyosin with respect to unregulated actomyosin.

An attempt to unify the observations of the present paper

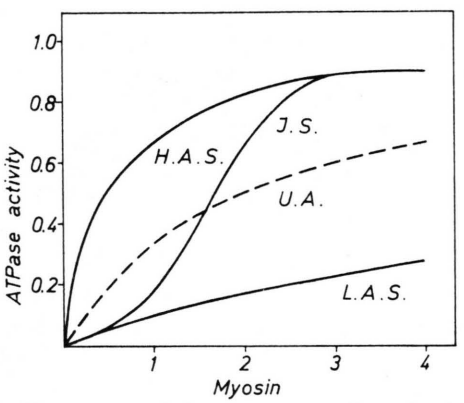

Fig. 7. The concept of the three states of regulated actin. The picture shows schematically the ATPase activity when different amounts of myosin (represented in arbitrary values) interact with regulated actin being in the high-affinity state (H.A.S.), with regulated actin being in the intermediate state (I.S.), or with regulated actin being in the low-affinity state (L.A.S.). For comparison the interaction of myosin with unregulated actin (u. A.) is shown.

Table I. ATPase activity of regulated and unregulated actomyosin.

Conditions under which ATPase activity of regulated actomyosin is (as compared to unregulated one) higher		lower	
Low ATP	High ATP	Low ATP	Hig ATP
Any ionic strength; Any A:M-ratio; Presence of Ca^{2+};	Low ionic strength and low A:M-ratio;	EGTA and high ionig strength; EGTA and high A:M-ratio;	EGTA; high ionic strength; high A:M-ratio;

A:M-ratio: ratio of actin to myosin.

Table II. The three postulated states of regulated actin and the conditions which favor them.

Low affinity state	Intermediate state	High affinity state
EGTA; High ATP (complete saturation of myosin with ATP = absence of rigor links);	Either EGTA and low ATP (incomplete saturation of myosin with ATP = presence of rigor links) or Ca^{2+} and high ATP (complete saturation of myosin with ATP = absence of rigor links).	Ca^{2+}; Low ATP (incomplete saturation of myosin with ATP = presence of rigor links);
Examples in: Fig. 2 B	Examples in: Fig. 1 A, B Fig. 2 A, B, Fig. 3 A–C, Fig. 5	Examples in: Fig. 1 C, Fig. 2 A, Fig. 4 A

The following hypothesis which is illustrated by Fig. 7 and Table II tries to incorporate the results of the present paper. It is assumed that regulated actin can adopt three states: A high-affinity state (myosin has a higher affinity to regulated than to unregulated actin), a low-affinity state (myosin has a lower affinity to regulated than to unregulated actin), and an intermediate state (myosin has a lower affinity to regulated than to unregulated actin when being present at low concentrations but a higher affinity to regulated actin when being present at higher concentrations). Hence, myosin interacts with the intermediate state of regulated actin in a cooperative way. However, it should be mentioned that cooperative binding alone is not sufficient to explain the obtained results because in this case increasing concentrations of regulated actin would only result in an ATPase activity which is lower than that of unregulated actomyosin but not, as it is in fact the case (*cf.* Figs 2, 3 A) in a decline of ATPase activity with increasing concentrations of regulated actin. Accordingly, one has to assume that the extent of enzymatic interaction between actin and myosin depends on the density of myosin on the actin filament.

Table II summarizes the conditions under which the different states of regulated actin occurred. From Table II (and diagrammatically from Fig. 7) it can be seen that the ATPase activity of actomyosin which contains regulated actin of the low-affinity state will be lower at all myosin concentrations than the ATPase activity of unregulated actomyosin whereas actomyosin with regulated actin in the high-affinity state has at all myosin concentrations a higher ATPase activity than unregulated actomyosin. Only when the intermediate state of regulated actin predominates (this is the case when conditions favoring the low-affinity state and those favoring the high-affinity state are both present thus "neutralizing" each other or when neither state is favored) myosin interacts with regulated actin in the described cooperative way. It is only in this case that the actin-myosin ratio has been found to be crucial.

This interpretation of the role of actin-myosin ratio differs from that of Weber and co-workers [6, 10, 11] probably because different phenomena have been observed. These workers have worked with HMM or with S-1 rather than with unfragmented myosin and they varied the actin-myosin ratio to a far larger extent than has been done in the present investigation. Weber and co-workers suggested that "rigor complexes" occurring between actin and HMM or S-1 unsufficiently saturated with ATP modulated the enzymatic behavior of the rest of the myosin molecules as long as both saturated and unsaturated myosin molecules are in contact with actin subunits belonging to one "functional unit" of regulated actin (seven actin monomers and one tropomyosin-troponin). When much more actin than myosin is present, the probability becomes low that more than one myosin molecule is in connection with one functional actin unit, hence cooperativity between the myosin molecules (resulting in a more efficient interaction between myosin and actin) which shall be mediated by the tropomyosin strand of the respective functional unit can no longer occur.

We too observed cooperativity of myosin towards actin only with regulated actin, but also at concentrations of ATP at which myosin can be expected to be fully saturated with ATP. (According

to a notion in their recent review Weber and Murray [6] observed a higher ATPase activity of regulated acto-S-1 at high ATP concentrations only when the concentration of S-1 was very high.)

Weber and Murray [6] also deduced three states of regulated actin. Their "off state" corresponds to our low-affinity state. However, we do not distinguish between "on state" and "potentiated" state but rather assume that potentiation of ATPase activity can occur when regulated actin is in the high-affinity state or in the intermediate state.

A possible mode of action of the different states of regulated actin

If myosin interacts with actin only in the form of preformed filaments, then only the spacing of these filaments on actin but not the spacing of the single myosin molecules can be determined by the actin-myosin ratio. If one, however, assumes that in the presence of ATP and during the interaction between actin and myosin not only a rearrangement between actin and myosin [12] but also desaggregation and reaggregation of myosin filaments takes place, then it is conceivable that this form of reaggregation may influence the ATPase activity of actomyosin (for the influence of ATP on myosin filaments see [23]). The difference in ionic strength dependence of the ATPase activity between actomyosin on the one hand and acto-HMM on the other hand as well as the lack of influence of the regulatory proteins on the cooperativity of the transition from high to low ATPase activity of acto-HMM (compare Fig. 1 and Fig. 4 B) suggests that the interaction between myosin and actin is not only determined by the properties of the myosin head but also by the "tail" of the myosin molecule (which is absent in HMM) and hence by the ability of the myosin molecule to form filamenteous aggregates. Accordingly the action of varying ionic strength may influence not only directly the interaction between actin and myosin but also by modulating the aggregation behavior of myosin during the actin-myosin interaction. There exists evidence [12] that actin promotes the formation of myosin filaments, one therefore may speculate that the difference in the ionic strength dependence between regulated and unregulated actomyosin may be due to different ways of forming myosin aggregates. If one assumes that myosin interacts with actin the better the more it has formerly been aggregated by actin one may further assume that the high-affinity state of regulated actin favors myosin aggregation whereas the low-affinity state provides less favorable conditions for myosin aggregation. One can further easily conceive that in the intermediate state the spacing of myosin on actin becomes crucial. When the intrinsic capacity of regulated actin to form myosin filaments lies between that of the low-affinity state and that of the high-affinity state (as it may be the case in the intermediate state) a critical population density of myosin on the actin filaments may be necessary for forming myosin filaments. Hence, filament formation "catalyzed" by the intermediate state of regulated actin may be low (resulting in a low ATPase activity of actomyosin) at a wide spacing of myosin on actin but may be high (resulting in a high ATPase activity of actomyosin) at a dense spacing of myosin on actin.

If one further assumes that the critical population density (which is thought to be necessary for the formation of myosin filaments) of myosin on regulated actin in the intermediate state may be influenced by ionic strength, the steep decline (Fig. 1 A) of the ATPase activity of regulated actomyosin (with regulated actin in the intermediate state) is easily explained.

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[1] W. Hasselbach, Biochim. Biophys. Acta **25**, 365 [1957].
[2] P. Dancker and W. Hasselbach, FEBS Letters **16**, 272 [1971].
[3] A. M. Gordon, R. E. Godt, S. K. B. Donaldson, and C. H. Harris, J. Gen. Physiol. **62**, 550 [1973].
[4] E. Eisenberg and C. Moos, Biochemistry **7**, 1486 [1968].
[5] S. Ebashi and M. Endo, Progr. Biophys. Mol. Biol. **18**, 123 [1968].
[6] A. Weber and J. M. Murray, Physiol. Rev. **53**, 612 [1973].
[7] P. Dancker, Proc. Int. Union Physiol. Sciences **9**, 129 [1971].
[8] M. Shigekawa and Y. Tonomura, J. Biochem. **71**, 147 [1972].
[9] W. Lehman and A. G. Szent-Györgyi, J. Gen. Physiol. **59**, 375 [1972].
[10] R. D. Bremel and A. Weber, Nature (New Biology) **238**, 97 [1972].
[11] R. D. Bremel, J. M. Murray, and A. Weber, Cold Spring Harbor Symp. Quant. Biol. **37**, 267 [1973].
[12] P. Dancker and M. Hoffmann, Z. Naturforsch. **28 c**, 401 [1973].
[13] D. J. Hartshorne and H. Mueller, Biochim. Biophys. Acta **175**, 301 [1969].
[14] S. E. Hitchcock, Biochemistry **12**, 2509 [1973].
[15] D. J. Hartshorne and P. Dreizen, Cold Spring Harbor Symp. Quant. Biol. **37**, 225 [1973].

[16] A. Weber and R. Herz, J. Biol. Chem. **238**, 599 [1963].
[17] A. G. Szent-Györgyi, Arch. Biochem. Biophys. **42**, 305 [1953].
[18] L. Leadbeater and S. V. Perry, Biochem. J. **87**, 233 [1963].
[19] E. Eisenberg and C. Moos, J. Biol. Chem. **242**, 2945 [1967].
[20] M. Bárány and K. Bárány, Cold Spring Harbor Symp. Quant. Biol. **37**, 157 [1973].
[21] J. R. Bendall, FEBS Letters **22**, 330 [1972].
[22] P. Dancker, Pflügers Arch. **315**, 187 [1970].
[23] H. Portzehl, Pflügers Arch. **272**, 463 [1961].