THE ULTRACENTRIFUGAL SEPARATION OF L-MYOSIN AND ACTIN IN AN ACTOMYOSIN SOL UNDER THE INFLUENCE OF ATP*

by

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INTRODUCTION

ATP induces marked changes in some physical properties of actomyosin sols such as viscosity[1], sedimentation rate[2,3] birefringence of flow[1] and light scattering[4,5,6]. The high intrinsic viscosity of actomyosin is decreased by ATP to a value close to that for L-myosin[3,7]. While at protein concentrations of 0.2 to 0.5 % actomyosin sediments in two or more peaks at 15 and more Svedberg units, ATP reduces the sedimentation constants to that of L-myosin[2,3]. Both these changes have been satisfactorily explained by assuming that ATP causes the dissociation of the complex actomyosin into L-myosin and actin, L-myosin representing $^2/_3$ to $^3/_4$ of the total protein[2,8,9,10]. The dissociation of actomyosin would explain the decrease in birefringence of flow which takes place on the addition of ATP. The changes of the ATPase activity which occur when actomyosin is brought from the gel into the sol form provide a further clue. Mg^{++} inhibits the dephosphorylation of ATP by L-myosin in both sol and gel form[8,11]. Actomyosin sol is similarly inhibited[8,13], but actomyosin gel is activated by Mg^{++}[8,11,12,13]. The contrast of the effect of Mg^{++} in sol and gel respectively is consistent with the view that in an actomyosin sol only L-myosin acts as an ATPase in consequence of the dissociation of actomyosin into L-myosin and actin[8,10].

It has been suggested[6] on the basis of light-scattering studies in presence and absence of ATP that ATP causes a change in shape of the actomyosin molecule rather than a dissociation. Using Zimm's extrapolation method the molecular weight was found[6] to remain constant after the addition of ATP. An increase in length and a "lateral inflation" of the molecules were postulated.

In order to obtain more direct evidence of the nature of the changes which ATP produces in an actomyosin sol, various fractions were separated from an ATP-actomyosin solution by ultracentrifugation. These have been studied with respect to properties which permit a differentiation between actomyosin, L-myosin and actin.

RESULTS AND DISCUSSION

Five different actomyosin preparations (0.6 M KCl, 10^{-3} M $MgCl_2$) containing 4 to 5×10^{-3} M ATP were centrifuged for 3 hours in an average gravitational field of

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Abbreviations used in this paper: ATP = adenosinetriphosphate, AMP = adenosinemonophosphate.

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100,000 g at 5° C. The initial protein concentration ranged from 0.3 to 0.6 %, the pH between 6.5 and 7. After the centrifugation the tubes contained a water clear solution and a small dense pellet. The top layers (40 to 50 % of the total volume) were separated from the rest of the solution and the pellet.

Centrifugation of an actomyosin solution in absence of ATP resulted in a quite different appearance. Here a water clear supernatant was separated by a sharp boundary from a turbid solution in the lower third of the tube and a large opaque precipitate was formed at the bottom.

On centrifugation after the addition of ATP the top layers contained between 20–30 % of the total amount of the protein present. This protein invariably exhibited properties characteristic of L-myosin but different from those of actomyosin.

1. *Sedimentation constant of the supernatant protein*

The sedimentation of the protein in the supernatant was determined after two phosphates had been split off the ATP. A single peak was found with a sedimentation constant $s_{20} = 6.0$. This value is in good agreement with those found for L-myosin[2,3,14,15] whereas the sedimentation constants reported[3] for fractionated actomyosin are above $s_{20} = 10$. Only in presence of ATP does actomyosin sediment with the same velocity as L-myosin[2,3] whereas when the ATP is dephosphorylated the higher sedimentation constants reappear and the low peak disappears[3]. The observation that in the isolated supernatant the low sedimentation remained after the ATP had been split suggests that the change in presence of ATP cannot be due to a change in shape of the actomyosin molecule under the influence of ATP. The fact that the sedimentation constant had the same value as that for L-myosin suggests that dissociation of actomyosin had occurred, only L-myosin remaining in the supernatant.

2. *Enzymic properties of the supernatant at low ionic strengths*

The principal difference in the enzymic behavior of L-myosin and actomyosin at ionic strengths below 0.1 resides in their reaction towards Mg^{++}. The ATPase activity of actomyosin is increased by Mg^{++} whereas that of L-myosin is inhibited[8,11,13].

When the ATPase activities of the supernatant and the corresponding actomyosin preparations were tested the following results were obtained. At an ionic strength below 0.1 and a Mg^{++} concentration of 10^{-3} M the rate of splitting of ATP by the supernatant was decreased to values between 0.005 and 0.02 μM/min/mg protein and amounted on the average to 5% of the activity of the corresponding actomyosin preparations (Table I). In contrast, 0.01 M Ca^{++} at an ionic strength below 0.1 activated the dephosphorylation by the supernatant to a rate similar to that of the corresponding actomyosin. With the various preparations the rate ranged from 0.25 to 0.71 μM/min/mg, the average value being 0.46 (Table I). A similar activity was found in samples of L-myosin. Therefore the depression of ATPase activity in the supernatant under the influence of Mg^{++} was caused not by a denaturation of the protein during ultracentrifugation but probably by the disappearance of actin from the actomyosin. The addition of actin to the supernatant increased its ATPase activity in presence of Mg^{++} more than tenfold. The same is known to occur when L-myosin is transformed into actomyosin by the addition of actin[13].

It should be pointed out, however, that the maximal activity (0.17 μM/min/mg)

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TABLE I

ATPASE ACTIVITY IN μmols/min/mg PROTEIN OF THE SUPERNATANT AND THE CORRESPONDING ACTOMYOSIN SOLUTIONS AT IONIC STRENGTHS BELOW 0.1 IN PRESENCE OF Ca^{++} OR Mg^{++}. pH BETWEEN 6.5–7.0

	0.01 M Ca^{++} Average of 5 prepns.	*0.001 M Ca^{++} Average of 3 prepns.*	*0.001 M Mg^{++} Average of 5 prepns.*
Supernatant	0.46	0.21	0.012
Actomyosin	0.44	0.27	0.22

achieved by the addition of actin (30% of the total protein) remained somewhat below that (0.21 μM/min/mg) of the corresponding actomyosin preparation. The discrepancy between the two preparations is even more striking when the activity of the supernatant + actin is compared with that of the corresponding actomyosin + actin instead of the actomyosin alone. Whereas the addition to actomyosin of as much actin as 20% of the total protein decreased its ATPase activity, it was increased from 0.21 to 0.28 μM/min/mg total protein by a small amount (5%) of actin. The difference between these two maximal values might have been due to a lower activity of the unpurified actin extract[16]. In addition, a small amount of a non-ATPase impurity might sediment together with L-myosin (see below).

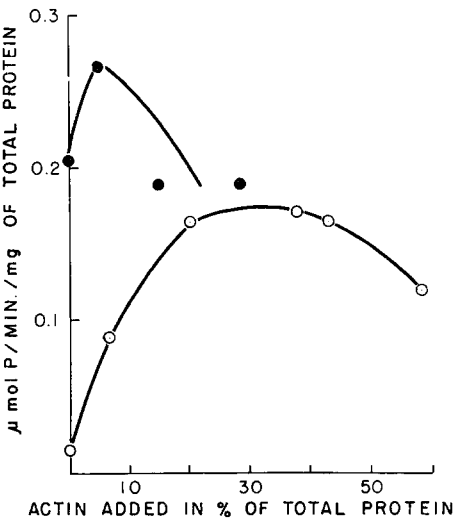

Fig. 1. The rate of splitting of ATP in dependence of the concentration of added actin ●●● to actomyosin, ○○○ to the supernatant. Ionic strength 0.07, $10^{-3} M$ Mg^{++}, pH 6.8.

3. *Solubility of the supernatant protein*

At an ionic strength below 0.1 and in presence of ATP actomyosin and L-myosin in low protein concentrations exhibit a significant difference in solubility. Both proteins precipitate very slowly and incompletely if the protein solutions are diluted from 0.1–0.2% protein in 0.6 M KCl to about 0.03% protein and an ionic strength below 0.1 in the absence of ATP. On the other hand, when ATP is added, actomyosin immediately begins to form a dense white precipitate[8]. In contrast, it has been reported[17] that an opalescent L-myosin solution clears up.

When the supernatants were diluted with water to an ionic strength of 0.08 to 0.06 they became opalescent. After the addition of ATP (final concentration $0.5–1 \cdot 10^{-3} M$) and in the presence of Mg^{++} ($10^{-3} M$) they cleared up immediately and sometimes stayed clear for as long as 12 hours. If actin (7 to 30% of the total protein) was first added, ATP caused immediate precipitation. Under similar conditions a L-myosin preparation was found to give similar reactions. In contrast actomyosin was immediately precipitated by ATP without the previous addition of actin.

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4. *"ATP-sensitivity"*

A third property which permits differentiation between actomyosin and L-myosin is the change in intrinsic viscosity produced by the addition of ATP[3,8,18]. The change has been expressed as "ATP sensitivity"[3]:

$$\frac{Z\eta - Z\eta ATP}{Z\eta ATP} \times 100; \quad Z\eta = \lim_{c \to 0} \frac{\eta spec}{c} = \frac{\ln \eta rel}{c}; \quad c = \text{mg protein/ml.}$$

The ATP sensitivity of L-myosin solutions is zero, of actomyosin solutions around 100.

The ATP sensitivity of the supernatant fractions was zero, that of the original actomyosin solutions varied between 90 and 146. The addition of actin to the supernatant increased the ATP sensitivity to a maximal value of 105 at an actin concentration of 40% of the total protein (Table II). The addition of actin to the corresponding

TABLE II

ATP SENSITIVITY OF THE SUPERNATANT AFTER ATP CENTRIFUGATION COMPARED TO THAT OF THE CORRESPONDING ACTOMYOSIN AFTER THE ADDITION OF ACTIN

	Actin added in per cent of the total proietn								
	0	*5*	*12*	*20*	*28*	*35*	*43*	*47*	*55*
Supernatant	0	8	22		84	90	103	101	97
Actomyosin	89	94	98	85					

actomyosin solution increased the activity from 90 to 100 after an actin addition of 13% of the total protein. Further addition of actin decreased the ATP sensitivity.

It appeared necessary to ascertain whether the L-myosin remaining in the supernatant might not have been a contaminant of the actomyosin preparation. This possibility was excluded as a major factor by the following observation. (i) Fractionation of the actomyosin preparations by KCl at concentrations between 0.2–0.3 *M* yielded no L-myosin. (ii) When actomyosin sols in absence of ATP were centrifuged for 3 hours at 100,000 *g* no more than 2 to 3% of the total amount of protein was found in the upper supernatant (40% of the total volume, as in the ATP experiments).

The values for enzymic activity recorded in Table III suggest that only about half of this small quantity of protein was L-myosin. The rates of dephosphorylation per mg protein activated by 0.01 *M* Ca^{++} or by a combination of Mg^{++} and actin were distinctly lower than those for the corresponding supernatant after centrifugation in presence af ATP.

Even this minor amount of L-myosin may not have been a contaminant of the

TABLE III

ATPASE ACTIVITY IN μmols/min/mg PROTEIN OF THE SUPERNATANT AFTER CENTRIFUGATION IN ABSENCE OF ATP (SUPERNATANT A) COMPARED TO THAT OF THE ATP-ACTOMYOSIN SUPERNATANT (SUPERNATANT B) AND THE CORRESPONDING ACTOMYOSIN.
Ionic strength below 0.1. pH between 6.5 and 7.

	0.01 M Ca^{++} Average of 2 prepns.	*0.001 M Mg^{++} Average of 2 prepns.*	*Actin + 0.001 M Mg^{++} Average of 2 prepns.*
Supernatant a	0.22	0.003	0.08
Supernatant b	0.5	0.01	0.17
Actomyosin	0.38	0.20	0.27

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actomyosin preparation. Its appearance in the supernatant could be attributable to partial dissociation of actomyosin in 0.6 *M* KCl[2,19]. This is suggested by the observation that the addition of actin to the actomyosin solution in an amount calculated to bind the L-myosin prior to centrifugation did not decrease the L-myosin concentration in the supernatant by more than the experimental error.

5. *Demonstration of the presence of actin in the bottom layers of the supernatant and in the pellet*

The lower half of the supernatant after centrifugation in presence of ATP contained actin in addition to myosin. This was demonstrated by precipitation and superprecipitation at ionic strengths below 0.1 by addition of ATP and by noting that the ATP was split in presence of Mg^{++}. However, the actin concentration was probably lower than in the original actomyosin solution. Whereas on the addition of actin, in an amount of 20% of the total protein, the rate of splitting of ATP by the original actomyosin was decreased from 0.2 to 0.19 μM phosphate/mg/min, it was increased in the bottom fraction from 0.12 to 0.16 μM/min/mg.

The pellets were washed with 0.6 *M* KCl and water in order to remove myosin and were then extracted with 10^{-4} *M* ATP. Very little protein could be extracted, most of the precipitate remaining insoluble, but this protein was found to react with the L-myosin in the supernatant similar to actin in the following ways. The extract itself did not precipitate on dilution and addition of ATP, but it caused the supernatant to precipitate immediately on the addition of ATP. It also increased the enzymic activity of the L-myosin supernatant in presence of Mg^{++} from 0.009 to 0.12 μ/min/mg. That only very little actin could be extracted after 3 hours of centrifugation at 100,000 *g* is not surprising since it is known that actin becomes sparingly soluble on centrifugation at 140,000 *g*[16].

6. *Degree of dissociation*

The isolation of L-myosin from the upper half of the supernatant and of actin from the pellet of actomyosin sols which were centrifuged at high speeds in presence of ATP shows that L-myosin and actin sediment separately. This can be explained only by dissociation of the complex under the influence of ATP. It cannot be concluded from these experiments that the dissociation is complete. However, the average value of the L-myosin concentration in the supernatant was 56% of the original protein concentration. 66-75% L-myosin would correspond to the total concentration present in actomyosin. In order to obtain an estimate how much the sedimentation of L-myosin itself would decrease the protein concentration in the supernatant, a preparation of L-myosin was centrifuged under similar conditions. The protein concentration in the supernatant was found to be 70% of the original. It may therefore be justifiably concluded that most of the protein was dissociated. Individual values, however, varied rather widely (between 35 to 70%), possibly in consequence of the agitation incident to the cessation of centrifugation and the removal of the tubes. It is not probable that the individual preparations dissociated to a significantly different degree, because the intrinsic viscosity of 4 actomyosin preparations in presence of ATP had closely similar values (between 0.17 and 0.18), only one preparation having a higher intrinsic viscosity (0.2).

Diagrams of actomyosin sedimenting in presence of ATP have been reported

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to show only the peak for L-myosin and not for actin[3,7]. In view of the above experiments it does not seem probable that all the actin had precipitated during acceleration since some actin was found in the lower layers of the supernatant after 3 hours at 100,000 *g*. Possibly the actin peak was, by reason of inhomogeneity, so much spread out as to be invisible, particularly as it was present in low concentration.

7. *Conclusions*

The finding that actomyosin dissociates in presence of ATP explains the results on viscosimetry, birefringence and the enzymic properties. It also explains why actin coupled with a fluorescent dye exhibits an equal depolarization of light in an actomyosin solution in presence of ATP as in an F-actin solution[20]. In absence of ATP the depolarization is higher in an actomyosin solution than in an F-actin solution[20].

The failure of light scattering[6] to reveal a change in molecular weight on the addition of ATP remains unexplained.

METHODS

1. *Protein preparations.* Actomyosin was prepared from rabbit muscle extracted for 24 hours according to PORTZEHL, SCHRAMM, WEBER[3]. The extracts were precipitated 2 to 4 times at 0.06 *M* KCl. Each preparation was fractionated once or twice at 0.2–0.3 *M* KCl in order to remove excess L-myosin[3]. In most cases the supernatant after centrifugation at 40,000–50,000 *g* in 0.6 *M* KCl was used. After centrifugation the turbidity of the solution was much decreased. However, the specific ATPase activity, the intrinsic viscosity and the ATP sensitivity were unchanged.

Actin was extracted according to STRAUB[21] from an acetone powder. I am grateful to Dr. A. G. SZENT GYÖRGYI for providing the acetone powder. The intrinsic viscosity of the water clear extract after polymerization was 0.17 and was increased after purification according to MOMMAERTS[16] (one cycle) to 0.36. Mainly the unpurified extract was used.

L-myosin was prepared according to PORTZEHL, SCHRAMM, WEBER[3].

2. *Sedimentation constant.* The sedimentation constant of the supernatant was determined in an analytical ultracentrifuge (Spinco) at 26° C. The solution contained 0.17 % protein, 0.6 *M* KCl, 10^{-3} *M* $MgCl_2$ $5 \cdot 10^{-3}$ *M* AMP, 0.01 *M* inorganic phosphate.

3. *Viscosity.* Viscosities were determined partly in an Ubbelohde and partly in an Oswald-Fenske viscosimeter. The solutions of actomyosin and those of L-myosin after the addition of actin showed thixotropy. The value after four consecutive runs was employed.

4. *ATPase activity.* The dephosphorylation of ATP was determined at temperatures varying between 15° to 20° C in different experiments. In addition to KCl and $MgCl_2$ or $CaCl_2$ (total ionic strength between 0.06 to 0.08) the solutions contained histidine (between 0.01–0.001 *M*). The pH was adjusted between 6.6 and 6.9. The rate of dephosphorylation was determined by measuring the increase of inorganic phosphate according to Fiske Subbarow.

ATP was obtained as dipotassium salt (Pabst).

SUMMARY

L-Myosin and actin have been separated from an actomyosin sol which had interacted with ATP.

When an actomyosin sol was treated with ATP and centrifuged at 100,000 *g*, the upper 40 % of the supernatant contained about 20 to 30 % of the total protein. The same actomyosin centrifuged in absence of ATP contained only 2 to 3 % of the total protein in the corresponding portion of the supernatant. This protein in the supernatant was identified as L-myosin by the following characteristics:

1. sedimentation constant after the dephosphorylation of ATP;
2. the inhibiting action of Mg^{++} on the ATPase activity, reversed by the addition of actin;
3. the disappearance of opalescence at an ionic strength below 0.1 on the addition of ATP and the immediate precipitation after the addition of actin;
4. an ATP sensitivity of zero, changing to one of 100 after the addition of an appropriate amount of actin.

The pellet produced on centrifugation yielded actin on extraction with water containing ATP.

From these experiments it is concluded that ATP effects the dissociation of an actomyosin sol.

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RÉSUMÉ

La L-myosine et l'actine ont été séparées à partir d'un sol d'actomyosine après interaction avec l'ATP.

Quand un sol d'actomyosine est traité par l'ATP et centrifugé à 100,000 *g*, les 40% supérieurs du surnageant renferment environ 20 à 30% de la protéine totale. La même actomyosine centrifugée en l'absence d'ATP renferme seulement 2 à 3% de la protéine totale dans la partie correspondante du surnageant. Cette protéine du surnageant a été identifiée à la L-myosine d'après 7 les critères suivants:

1. constante de sédimentation après déphosphorylation de l'ATP;
2. action inhibitrice de Mg^{++} sur l'activité ATPasique, réversible en présence d'actine;
3. disparition de l'opalescence à une force ionique inférieure à 0.1 quand on ajoute de l'ATP et précipitation immédiate après addition d'actine;
4. sensibilité à l'ATP égale à zéro, devenant égale à 100 après addition d'une quantité convenable d'actine.

L'extraction par une solution aqueuse d'ATP du culot obtenu par centrifugation fournit de l'actine.

De ces expériences les auteurs concluent que l'ATP provoque la dissociation d'un sol d'actomyosine.

ZUSAMMENFASSUNG

L-Myosin und Aktin wurden aus einem Aktomyosin-Sol isoliert, welcher mit ATP reagiert hatte.

Ein Aktomyosin-Sol wurde mit ATP behandelt und bei 100,000 *g* ausgeschleudert. Die oberen 40% der überstehenden Flüssigkeit enthielten ungefähr 20–30% des totalen Proteins. Dasselbe, in Abwesenheit von ATP ausgeschleuderte Aktomyosin enthielt nur 2–3% des totalen Proteins in der entsprechenden Portion der überstehenden Flüssigkeit. Dieses Protein in der überstehenden Flüssigkeit wurde mit Hilfe seiner folgenden charakteristischen Eigenschaften als L-Myosin erkannt:

1. Sedimentierungskonstante nach Dephosphorylierung von ATP;
2. die hemmende Wirkung von Mg^{++} auf die ATPase-Aktivität, welche durch die Hinzufügung von Aktin rückgängig gemacht wurde;
3. Das Verschwinden der Opalescenz bei Ionenstärke unter 0.1, falls man ATP hinzufügte, und der sofortige Niederschlag nach Hinzufügung von Aktin;
4. Eine ATP-Empfindlichkeit gleich Null, welche nach Hinzufügung einer entsprechenden Aktin-Menge auf 100 anwuchs.

Durch Extraktion mit ATP enthaltendem Wasser konnte Aktin aus dem Zentrifugationsrückstand gewonnen werden.

Es wird aus diesen Versuchen gefolgert, dass ATP die Dissoziation eines Aktomyosin-Sols hervorruft.

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