Calcium-Dependent Myosin
from Insect Flight Muscles

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SUMMARY Calcium regulation of the insect actomyosin ATPase is associated with the thin filaments as in vertebrate muscles, and also with the myosin molecule as in mollusks. This dual regulation is demonstrated using combinations of locust thin filaments with rabbit myosin and locust myosin with rabbit actin; in each case the ATPase of the hybrid actomyosin is calcium dependent. The two regulatory systems are synergistic, the calcium dependency of the locust actomyosin ATPase being at least 10 times that of the hybrid actomyosins described above. Likewise Lethocerus myosin also contains regulatory proteins. The ATPase activity of Lethocerus myosin is labile and is stabilized by the presence of rabbit actin. Tropomyosin activates the ATPase of insect actomyosin and the activation occurs irrespective of whether the myosin is calcium dependent or rendered independent of calcium.

INTRODUCTION

Muscular contraction in all animals appears to be regulated by the concentration of calcium within the myofibrils. The calcium interacts with so-called regulatory proteins which in the vertebrates (Ebashi and Endo, 1968) and in the arthropods (Regenstein, 1972; Bullard et al., 1973; Lehman et al., 1973) are a complex of troponin and tropomyosin occurring on the thin filament. Troponin is a complex molecule itself and one of its components interacts with calcium specifically (Hartshorne and Mueller, 1968; Schaub and Perry, 1969; Greaser and Gergely, 1971). In mollusks, on the other hand, no troponin is found and the regulatory system is linked to the myosin and calcium binds to this molecule (Kendrick-Jones et al., 1970). Apparently in all these cases, the calcium, by combining with the regulatory proteins, modifies the affinity of myosin for actin (Eisenberg and Kielley, 1970; Parker et al., 1970; Lehman and Szent-Györgyi, 1972 a; Szent-Györgyi et al., 1973) and this controls the adenosine triphosphatase (ATPase) activity and the force of contraction (Helm and Podolsky, 1969; Podolsky and Teichholz, 1970; Julian, 1971; Abbott,
1973). The regulatory proteins act by inhibiting the actin-activated ATPase of myosin when calcium is absent. Remarkably, annelids and certain groups of arthropods contain both thin filament-linked and myosin-linked regulatory systems within the same muscles (Lehman et al., 1973; Lehman and Szent-Györgyi, unpublished results).

In most cases this dual nature of regulation has been demonstrated by studying the interaction of isolated thin filaments with rabbit myosin and by use of an indirect ATPase test (Lehman et al., 1973). In this indirect assay we introduce pure rabbit actin to myofibrils or natural actomyosin at low calcium concentration, and determine if the myosin present is blocked or is free to interact with the rabbit actin. The advantage of this “competitive actin-binding assay” is that it tests whether the myosin is calcium dependent or not, without having to extract or purify the myosin. Interpretation of the results of this assay does, however, assume firstly that in all muscles actin and myosin are dissociated in the absence of calcium, and secondly that any actin (or actin complexed with tropomyosin) will activate the ATPase of a particular myosin.

The competitive actin-binding assay indicates that several orders of insects contain a myosin-linked regulatory system in addition to the thin filament-linked system (Lehman et al., 1973; Lehman and Szent-Györgyi, unpublished results). There is, however, as yet no evidence on purified myosin from insects being calcium dependent. In the present investigation myosin was prepared from locust and Lethocerus, and direct evidence is presented for a myosin-linked regulatory system in these insects.

**MATERIALS AND METHODS**

**Preparations**

All proteins were prepared at 0–4°C. Rabbit actin free of tropomyosin was prepared by the method of Straub (1942) employing either of two modifications (Kendrick-Jones et al., 1970; Bullard et al., 1973). Pure tropomyosin was prepared by the method of Bailey (1948) with the additional purification procedure of Hartshorne and Mueller (1969) or Lehman and Szent-Györgyi (1972a). Rabbit myosin was prepared according to Szent-Györgyi (1951) with the modification of Mommaerts and Parrish (1951) which further removes actomyosin impurities. Insect myofibrils were prepared and washed as described by Bullard et al., (1973) from the flight muscles of the locust, Schistocerca gregaria, and the waterbug, Lethocerus cordofanus.

**LOCUST MYOSIN**

Locust myosin was prepared directly from washed myofibrils by a modification of Weber’s procedure for dissociating actin and myosin in the presence of ATP (Weber, 1956). The washed myofibrils were suspended in 50 mM KCl, 1 mM MgCl₂, 5 mM Na⁺ or K⁺ phosphate buffer (pH 7.0). The KCl concentration was then increased to 0.6 M KCl and ATP was added (final concentration 1 mM, pH 7.0). The suspension was homogenized with a Potter homogenizer (Potter Instrument Co. Inc., Melville, N.Y.) and immediately sedimented for 10 min at 30,000 g to
remove coarse fragments, and then at 200,000 g for 2½ h to remove the remaining actin. The supernatant containing myosin and paramyosin was used in these studies. No attempt was made to separate the myosin from paramyosin. Some preparations of the myosin were precipitated by dialyzing against 50 mM KCl, 1 mM MgCl₂, 5 mM Na⁺ or K⁺ phosphate buffer (pH 7.0); very little locust myosin is precipitated by rapid dilution of the KCl to 30 mM using phosphate buffer (5 mM, pH 6.0–7.0). Preparations of the extracted myosin could be precipitated by dilution, if the myosin first was mixed with rabbit actin. Here rabbit actin and the locust myosin were mixed and then diluted with 20 vol of 50 mM NaCl, 1 mM MgCl₂, 5 mM phosphate buffer, pH 7.0. The mixture was immediately centrifuged for 5 min at 20,000 g and the pellet suspended in the same solution.

**Locust Thin Filaments** Flight muscles were glycerinated for 12–36 h while still attached to the cuticle, in a solution consisting of 100 mM KCl, 5 mM MgCl₂, 5 mM Na⁺ or K⁺ phosphate buffer, pH 7.0 and 50% glycerol (vol/vol). The muscles were then dissected and rinsed in 40 mM KCl, 1 mM MgCl₂, 5 mM Na⁺ or K⁺ phosphate buffer, pH 7.0, and homogenized in a VirTis homogenizer (VirTis Co. Inc., Gardiner, N.Y.) in the same solution. The myofibrils were sedimented for 5 min at 2000 g. The pellets were resuspended in a solution consisting of 100 mM KCl, 5 mM MgCl₂, 0.1 mM EGTA, 5 mM Na⁺ or K⁺ phosphate buffer, pH 6.0; the resuspension and sedimentation was repeated twice. The washed myofibrils were then resuspended in the same solution containing in addition 5 mM ATP (pH 6.0) and homogenized using a Potter homogenizer. The suspension was sedimented for 10 min at 30,000 g to remove coarse fragments, and for an additional 30 min at 80,000 g to remove thick filaments. The thin filaments were then collected by sedimenting the supernatant at 80,000 g for 2½ h and resuspended in 40 mM KCl, 1 mM MgCl₂, 5 mM Na⁺ or K⁺ phosphate buffer, pH 7.0.

**Lethocerus Myosin** *Lethocerus* myosin was extracted by the method used for locust. Preparations were also made of *Lethocerus* myosin and rabbit actin where the myosin was extracted in the presence of rabbit actin and the native thin filaments selectively removed. This was done to protect the ATPase activity of the myosin. In this preparation, washed myofibrils from about 7 g of muscle were sedimented at 2,500 g for 15 min and resuspended in 1 vol of 1.4 M KCl, 2 mM MgCl₂, 20 mM K-phosphate buffer (pH 7.0) to which was added another volume of 0.7 M KCl, 1 mM MgCl₂, 10 mM K-phosphate buffer (pH 7.0); ATP was then added (final concentration 1 mM, pH 7.0). Pure rabbit actin was added to give a concentration of about 1 mg/ml in the extract. The mixture was stirred for 6–8 min and centrifuged at 15,000 g for 15 min producing a hard pellet. The supernatant was carefully removed with a Pasteur pipette and filtered through glass wool to remove lipid. The rabbit actin was not removed from the extract because if it is, the myosin is inactivated. The protein concentration of the extract was about 4 mg/ml.

Some extracts were precipitated by dialyzing against 40 mM KCl, 1 mM MgCl₂, 1 mM ATP, 10 mM K-phosphate buffer (pH 7.0). The precipitate was sedimented at 2,500 g for 10 min and dissolved in 0.6 M KCl, 1 mM MgCl₂, 1 mM ATP, 10 mM K-phosphate buffer (pH 7.0).
Analysis

The Mg-activated ATPase of actomyosin was measured using the pH-stat method as previously described (Szent-Györgyi et al., 1971); ATPase assays were performed at 25°C. A creatine phosphate-creatine kinase backup system was not employed. Calcium sensitivity was measured by comparing the ATPase rates in the presence of 0.1 mM EGTA before and after calcium was added to 0.6 - 1.0 × 10^{-4} M. The substrate was Mg-ATP and the initial concentrations were 0.7 mM ATP and 1 mM MgCl₂. Protein samples dissolved in approximately 0.6 M KCl were added to 10 ml of assay solution and the final salt concentration in the assay medium varied between 20 and 40 mM. The only pH buffers present were the ATP and EGTA and 0.05 - 0.35 mM phosphate introduced with the sample.

Protein concentrations were measured by the method of Lowry et al., (1951) and by the microbiuret method (Goa, 1953), each standardized by Kjeldahl N-determinations. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed using Coomassie Blue as a stain according to Weber and Osborn (1969). Stained gels were scanned with a Gilford densitometer attachment (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) on a spectrophotometer.

RESULTS AND INTERPRETATION

Locust Flight Muscle

Locust Myosin and Rabbit Actin  The locust myosin is relatively pure as shown by the presence of only trace amounts of actin and tropomyosin on SDS gels (Fig. 1a) and by the very low Mg-activated ATPase of the extracted myosin (Table I, a) which is stimulated by pure rabbit actin about 20 times (Table I, b). Myosin can be extracted directly from the myofibrils because most of the thin filaments remain firmly attached to the Z disks and are sedimented with them; the remaining thin filaments are removed by high speed centrifugation.

The Mg-ATPase of the locust myosin stimulated by rabbit actin is dependent on calcium concentration (Table I, b). Since the actin does not contain regulatory proteins, the calcium sensitivity can be attributed to the locust preparation. Tropomyosin activates the ATPase rate of the locust actomyosin about three times, and the activation at low and high calcium concentrations is about the same (Table I, c); thus, the degree of the calcium dependence is not altered by tropomyosin. The ATPase activity of locust myosin in the presence of both actin and tropomyosin is lower than the ATPase activity of locust myofibrils which is about 0.3 μmol ATP split/min/mg myofibrillar protein at the high calcium concentration.

Locust myosin which has been precipitated and redissolved and then is mixed with rabbit actin, has a normal ATPase activity but it is not calcium dependent. Thus, this reprecipitation results in a loss of calcium sensitivity. Since the entire dialyzed sample of precipitated myosin was assayed without


**TABLE I**

**ATPASE ACTIVITIES OF LOCUST PREPARATIONS**

| No. of preparations | Mg-ATPase | Calcium ATPase | Calcium sensitivity ATPase (calcium)/ATPase (EGTA) |
|----------------------|-----------|----------------|-------------------------------------------------|
| (a) Locust myosin alone | 6 | — | 0.004 | 2.3 (1.8-3.4) |
| (b) Locust myosin + rabbit actin‡ | 6 | 0.039 | 0.085 | 2.4 (2.1-3.8) |
| (c) Locust myosin + rabbit actin + rabbit tropomyosin§ | 4 | 0.11 | 0.26 | 2.4 (2.1-3.8) |
| (d) Locust myofibrils|| 3 | 0.06 | 0.29 | 5 |

* ATPase activities of myofibrils calculated in terms of total protein present, the other values are in terms of myosin present. The results are averages of the number of preparations indicated. Figures in brackets are the range of values observed. The duration of an assay was from 4 to 12 min; the ATP being split did not exceed 60% of that added.

‡ Myosin and actin were mixed in ratios of 3-4/1 (wt/wt). The calcium sensitivity of every myosin preparation was also tested with actin in concentrations two and four times higher than saturating amounts, and was found to be unchanged.

§ The ratio of actin to tropomyosin was 2/1 (wt/wt).

‖ The ATPase of locust myofibrils in EGTA was high for the first 2 min of any assay and then lowered. The rate in EGTA was measured after 10 min in the EGTA and compared with the initial rate with added calcium.

Further fractionation, the loss of the calcium dependence is not due to removal of a factor. The ATPase of this preparation is still activated to the same extent by tropomyosin, and thus this property of the myosin is not influenced by precipitation. When the same myosin preparation is not precipitated by itself but precipitated in the presence of rabbit actin and then collected by centrifugation, it retains its calcium-dependent properties (Table II). In this procedure the myosin is coprecipitated with various excess concentrations of actin, insuring that the myosin will bind saturating amounts of actin. Therefore, actin prevents the loss of the calcium dependence during precipitation of the myosin.

SDS gels of such coprecipitates of locust myosin and rabbit actin show large excesses of actin to tropomyosin (Fig. 1b). This further illustrates that the observed calcium dependence of locust myosin and rabbit actin is not due to contaminating tropomyosin.

Tropomyosin is a necessary constituent for the actin-linked regulatory system in the arthropods as it is in the vertebrates (Regenstein, 1972; Lehman et al., 1973; Bullard et al., 1973). Since the evidence is that locust myosin preparations have a calcium-dependent ATPase in the absence of tropomyosin, this shows that the calcium sensitivity is an inherent property of the myosin.

**LOCUST MYOSIN AND LOCUST THIN FILAMENTS** Locust thin filaments confer a calcium dependence to the actomyosin ATPase formed with rabbit myosin.
Figure 1. 10% SDS polyacrylamide gels of insect muscle preparations. Bands seen on these gels correspond to the myosin heavy chain (HC), paramyosin (P), a 55,000 dalton protein (X) associated with arthropod thin filaments, actin (A), tropomyosin (TM), and the myosin light chains (LC). (a) Unprecipitated locust myosin. (b) Locust myosin co-precipitated with rabbit actin. N.B. Even though this gel shows a heavy loading of actin, the actin band has not superimposed on the tropomyosin band which appears as an extremely faint line. (c) Locust thin filaments. N.B. SDS gels of all other arthropods thin filaments show significant amounts of 19,000, 29,000, and 55-60,000 dalton chains. Preparations of locust thin filaments, whether from fresh or glycerinated muscles, only show the 55,000 dalton chain and trace amounts of the 19,000 dalton chain. Locust thin filaments always form a calcium-sensitive ATPase with rabbit myosin, and it may be that the troponin chains have been partly proteolyzed during preparation. (d) Lethocerus myosin extracted in the presence of rabbit actin.

### Table II

| Ratio (wt/wt) of actin added to myosin | 1.4 | 2.0 | 2.1 | 4.2 |
|---------------------------------------|-----|-----|-----|-----|
| Calcium sensitivity                   | 3.0 | 2.4 | 2.3 | 2.2 |
| ATPase (calcium)/ATPase (EGTA)        |     |     |     |     |

These data are results from three different preparations of myosin. Since the percentage of myosin in the pellet was not determined, specific activities are not given.

(Table III). Since locust muscle is a tissue from which calcium-sensitive thin filaments and myosin can be prepared, it is of interest to compare their individual and combined contributions to the calcium sensitivity of the muscle. This was examined by mixing locust thin filaments with calcium insensitive rabbit myosin, and locust myosin with calcium-insensitive pure rabbit actin, and comparing the calcium sensitivities of these actomyosins with that of locust thin filaments and locust myosin. The calcium sensitivity of the mixture of locust thin filaments and locust myosin is greater than either of the other two actomyosins by a factor of about 10 (Table III).
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**TABLE III**

ATPase Activities Conferred by Locust Thin Filaments and Locust Myosin

|                      | Mg-ATPase | Calcium sensitivity |
|----------------------|-----------|---------------------|
|                      | EGTA      | Calcium/ATPase (EGTA) |
| Rabbit actin + rabbit myosin, calcium-insensitive control proteins | 0.5 0.5 | 1.0 |
| Rabbit actin + locust myosin* | 0.023 0.083 | 3.4 |
| Locust thin filaments + rabbit myosin | 0.1 0.5 | 5 |
| Locust thin filaments + locust myosin | <0.008† 0.31 | >40 |

* The preparation of locust myosin used in this experiment showed the highest calcium sensitivity of all preparations examined.
† The ATPase rate in EGTA was lower than could be measured.

**Lethocerus Flight Myosin**

Extracted *Lethocerus* myosin has a low ATPase activity that is activated by rabbit actin and tropomyosin (Table IV, b and c). But although this activated ATPase is sensitive to calcium, the specific activity is never greater than 0.05 μmol ATP split/min/mg myosin, a value considerably less than that of myofibrils. The low ATPase is likely to be a result of the isolation of the myosin in 0.6 M KCl, since unfractionated extracts of myofibrils are fully active. Thus, it seems likely that the myosin is inactivated by removal of the thin filaments.

With this idea in mind, myosin was extracted from myofibrils in the presence of rabbit actin and indeed the preparation is more active and is calcium sensitive (Table IV, d and e). The extract contained myosin (heavy and light chains) and actin, and a small amount of tropomyosin, and a protein of subunit molecular weight 55,000 (Figs. 1d, 2). The actin band is mostly due to the rabbit actin. This is evident from the densitometry tracing; the ratio of actin to tropomyosin in the extract is about 15:1 (wt/wt) which is considerably higher than the 3:1 (wt/wt) found with *Lethocerus* myofibrils.

The ATPase of this actomyosin does not seem to be influenced by the small amounts of intrinsic tropomyosin since addition of rabbit tropomyosin activates the ATPase rate (Table IV, e) to the same extent as that obtained previously with *Lethocerus* myosin free of tropomyosin (Bullard et al., 1973). Since this actomyosin has a calcium-dependent ATPase and most of the actin is uncomplexed with tropomyosin, the calcium sensitivity therefore is very likely to be a property of the myosin, for the same reasons discussed in the case of the locust.

The calcium dependence of *Lethocerus* myosin is likewise lost by precipitation of the preparation; this occurs in the presence of rabbit actin. The precipitation also results in the specific ATPase activities being increased from three to four times (Table IV, f and g).
**TABLE IV**

ATPASE ACTIVITIES OF *LETHOCERUS* PREPARATIONS

| No. of preparations | EGTA | Calcium | ATPase (Calcium)/ATPase (EGTA) |
|----------------------|------|---------|---------------------------------|
|                      | μmol ATP split/min/mg* |         |                                  |
| (a) *Lethocerus* myofibrils | 2 | 0.08 | 0.25 | 3.1 |
| (b) *Lethocerus* myosin extracted alone | | | | |
| (c) *Lethocerus* myosin extracted alone, then rabbit actin + rabbit tropomyosin added‡ | 5 | 0.008 | 0.04 | 5 |
| (d) *Lethocerus* myosin extracted with rabbit actin‡ | 4 | 0.014 | 0.08 | 2.7 | (2.5-3.0) |
| (e) *Lethocerus* myosin extracted with rabbit actin, then rabbit tropomyosin added‡ | 4 | 0.029 | 0.10 | 3.4 | (2.8-4.0) |
| After precipitation: | | | | |
| (f) *Lethocerus* myosin extracted with rabbit actin, then precipitated | 2 | 0.18 | 0.17 | 0.94 |
| (g) *Lethocerus* myosin extracted with rabbit actin, then precipitated and rabbit tropomyosin added | 2 | 0.40 | 0.33 | 0.83 |

* ATPase activities of myofibrils are calculated in terms of total protein, other values in terms of myosin only. The results are averages of the number of preparations indicated. Figures in brackets are ranges of values.

‡ Myosin and actin were mixed in 0.5 M KCl in the ratio of 3/1 (wt/wt), actin and tropomyosin in the ratio 2/1 (wt/wt).

§ Specific activities calculated assuming that the difference between the total protein concentration of the extract and the rabbit actin was entirely *Lethocerus* myosin, i.e. 1 mg/ml actin, 3 mg/ml myosin. Densitometry of 7.5% SDS gels of extracts of *Lethocerus* myosin with rabbit actin, show that the ratio of total protein to actin is about 4:1. The real specific activities are probably slightly higher since other proteins in the extract such as paramyosin have been included in calculating the myosin fraction.

Myosin preparations extracted with rabbit actin had saturating amounts of actin. The ATPase activity and calcium sensitivity of the myosin are not significantly altered by actin concentrations up to four times that in the extract.

**DISCUSSION**

It was previously shown that, like the vertebrates, the insects contain a thin filament-linked regulatory system (Maruyama et al., 1968; Meinrenken, 1969; Lehman and Szent-Györgyi, 1972; Bullard et al., 1973). However, no attempts were made to look for a myosin-linked system until the competitive actin-binding test was developed, and it was then found that insects, like annelids, contain a dual regulatory system (Lehman et al., 1973). In the present study we have shown directly that myosin from insects can confer calcium sensitivity to an actomyosin ATPase. Although our myosin preparations contain tropomyosin contamination, tropomyosin is not present in sufficient amounts to be considered part of a relaxing protein system.

The reason for the loss in calcium sensitivity after insect myosin is precipitated is not known. Whereas with scallop myosin, removal of a myosin light
chain was shown to be the cause (Szent-Györgyi et al., 1973); there is, however, no obvious loss of light chain components after precipitation of locust myosin and this agrees with results reported previously for myosins of other insects (Bullard et al., 1973). Although we cannot explain why insect myosin loses its calcium-dependent properties after precipitation, or how actin protects the myosin, our results do explain why in a previous study (Bullard et al., 1973), where only precipitated myosin was analyzed, calcium sensitivity was not detected.

An advantage of having both the thin filament and myosin-linked systems in a single muscle is obvious. Since both systems probably act by independent mechanisms (Lehman et al., 1973), the calcium sensitivity of the dual system should be a function of the product of the individual sensitivities and thus increased. If the interaction behaved as a monomer-monomer reaction, the increased calcium sensitivity would be expected to be the product of the individual calcium sensitivities. The increased sensitivity is in fact even greater than this. If a double system is a more powerful way to control muscular contraction, then it is unclear why certain groups evolved single systems, and if in these systems there are further adaptations.

Tropomyosin has been found to activate the ATPase of Limulus (Lehman and Szent-Györgyi, 1972a) and all insect muscles investigated (Bullard et al., 1973) but this property is not a general feature of arthropods since no crusta-
cean investigated demonstrates the effect (Lehman and Szent-Györgyi, unpublished results; Regenstein, 1972). The most likely explanation for the activation is that tropomyosin provides a better fit for particular myosins on actin (Lehman and Szent-Györgyi, 1972 a).

Preparations of relaxing proteins have been found to activate the actomyosin ATPase of vertebrate muscles (Katz, 1964; Schaub et al., 1967; Hartshorne and Mueller, 1968; Greaser and Gergely, 1971; Spudich and Watt, 1971). This has never been investigated in detail, and it is not understood which component is responsible for the activation. At low ATP concentrations, it has been observed (Shigekawa and Tonomura, 1972; Bremel et al., 1973) that the ATPase of actomyosin or an actin-subfragment-1 system is activated by tropomyosin alone. Bremel et al. (1973) suggest that tropomyosin mediates a cooperative change on the actin filament, resulting in a greater reactivity of the actin for myosin. The activation by tropomyosin found in Limulus and the insects may involve a similar type of cooperativity occurring at higher ATP concentration. Further research should establish if activation by tropomyosin is restricted only to certain species, and what its function in the regulation of muscular contraction is.

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