The Contractile and Regulatory Proteins of Insect Flight Muscle

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1. Myosin, actin and the regulatory proteins were prepared from insect flight muscle. 2. The light subunit composition of the myosin differed from that of vertebrate muscle myosin. The ionic strength and pH dependence of the myosin adenosine triphosphatase (ATPase) were measured. 3. Actin was associated with a protein of subunit molecular weight 55000 and was purified by gel filtration. Impure actin had protein bound at a periodicity of about 40 nm. 4. Regulatory protein extracts had tropomyosin and troponin components of subunit molecular weight 18000, 27000 and 30000. Crude extracts of regulatory proteins inhibited the ATPase activity of desensitized or synthetic actomyosin; this inhibition was relatively insensitive to high Ca^{2+} concentrations. Purified insect regulatory protein produced as much sensitivity to Ca^{2+} as did the rabbit troponin-tropomyosin complex. 5. Synthetic actomyosins were made from rabbit and insect proteins. Actomyosins containing insect myosin had a low ATPase activity that was activated by tropomyosin. The Ca^{2+} sensitivity of actomyosins containing insect myosin or actin, with added troponin-tropomyosin complex from rabbit, was comparable with that of rabbit actomyosin.

The fibrillar flight muscle of insects has oscillatory mechanical activity that is not due to activation by the sarcotubular system but is a property of the contractile proteins (Jewell & Rüegg, 1966). Glycerinated fibres of fibrillar muscle require Ca^{2+} for activity, as do fibres of non-fibrillar muscle, but they can be further activated by a small amount of stretching (Rüegg & Tregear, 1966; Chaplain, 1967). Studies on proteins isolated from fibrillar muscle (Kominz, *et al.*, 1962; Maruyama, 1965) have shown that the major proteins of the myofibril are similar to those of vertebrate striated muscle. However, there have been reports that myofibrils and actomyosin from fibrillar muscle are activated less by Ca^{2+} than are the same preparations from non-fibrillar muscle (vom Brocke, 1966; Maruyama *et al.*, 1968). The gradation of ATPase† with increasing Ca^{2+} concentration is partly due to a troponin-like protein fraction (Maruyama *et al.*, 1968) which is essential for oscillatory activity (Meinrenken, 1969). Also Lehman *et al.* (1973) found evidence for a dual system of Ca^{2+} regulation in *Lethocerus* muscle, associated with the thin filaments and with myosin.

The aim of the present investigation was to determine what differences there were between the insect and rabbit contractile proteins which might be relevant to the mechanism of stretch-activation. We

attempted to do this by isolating myosin, actin and troponin and reconstituting actomyosin with the troponin-tropomyosin complex, by using insect and rabbit components. A brief account of this work was presented at the 4th International Biophysics Congress, Moscow, August 1972.

Methods

Material

The dorsal longitudinal and oblique flight muscles were dissected from the water bugs, *Lethocerus cordofanus* and *L. maximus*, and the dung beetle *Helicopris japedus*.

Myofibrils

All the procedures described were performed at 0–4°C. Myofibrils were prepared by homogenizing the muscle with a Virtis homogenizer in 50 vol. of a solution containing 0.3 M-sucrose, 0.1 M-KCl, 0.01 M-potassium phosphate buffer (pH 7.0), 1 mM-MgCl₂, 1 mM-EGTA [ethanedioxybis(ethylamine)tetra-acetate] and 0.01 M-sodium azide for about 1 min at top speed. The myofibrils were washed by centrifuging them at 600g and resuspending them in the same solution. The washing removed mitochondria, which remain in the supernatant sucrose solution (Abbott & Chaplain, 1966), and large amounts of lipid and trachea, which float. The procedure was repeated six times or until washings were only faintly yellow and

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† Abbreviation: ATPase, adenosine triphosphatase.

examination in the light microscope showed the myofibrils were relatively free of mitochondria. Before extracting them further the myofibrils were washed three times by centrifuging them and then resuspending them in 0.1 M-KCl-0.01 M-potassium phosphate buffer (pH 7.0).

Desensitized myofibrils and actomyosin

Insect myofibrils that had been extracted for regulatory protein with 50 mM-KCl, 20 mM-Tris-HCl (pH 8.0) and 2 mM-dithiothreitol as described below, were washed with the same solution until the ATPase was insensitive to the concentrations of Ca^{2+} in the range 1 nM to 10 μM .

Desensitized actomyosin from the rabbit was prepared by the method of Schaub & Perry (1971).

Myosin

Rabbit myosin was extracted from minced muscle with Guba-Straub solution and purified as described by Mommaerts (1958).

Insect myosin was extracted from washed myofibrils with Hasselbach-Schneider-Zebe solution containing 1 M-KCl, 0.01 M-sodium pyrophosphate, 1 mM-MgCl₂ and 0.02 M-potassium phosphate buffer (pH 6.5) (Bullard & Reedy, 1973). Myofibrils from 5–25 g of muscle were sedimented, extracted for 10–15 min with about 3 vol. of solution and the residue was removed by centrifugation at 10000g for 30 min. The supernatant was filtered through glass wool to remove floating lipid and dialysed for about 16 h against 0.25 M-KCl, adjusted to pH 6.5 with 10 mM-NaHCO₃. The precipitate of actomyosin was sedimented by centrifuging at 50000g for 30 min. The supernatant was then dialysed for 3–16 h against 0.03 M-KCl, adjusted to pH 6.5 with 10 mM-NaHCO₃, and the precipitated myosin was spun down at 1000g for 30 min, resuspended in a small volume of 2 M-KCl (adjusted to pH 7.0 with 10 mM-NaHCO₃) and diluted to make the final KCl concentration 0.5 M. Where indicated 2 mM-dithiothreitol or 14 mM-2-mercaptoethanol were included in all the solutions used for preparing myosin. The myosin was clarified by centrifuging at 50000g for 1 h. The average yield was 2.3 mg from 1 g of muscle.

Actin

Rabbit actin was prepared as described by Seraydarian *et al.* (1967) except that the actin was stirred for 1 h in 0.6 M-KCl, 1 mM-MgCl₂ for the second cycle of polymerization. This procedure removed tropomyosin (Spudich & Watt, 1971).

Acetone-dried insect muscle was prepared as follows from the residue obtained after extracting myosin. The residue was washed by resuspension in

5 vol. of 0.05 M-KCl, adjusted to pH 7.5 with 10 mM-NaHCO₃, stirred for 15 min, and then centrifuged at 1000g for 20 min. The washing was repeated twice with 10 vol. of solution each time. If a solution of lower ionic strength was used the muscle residue swelled and large amounts of protein were extracted. The washed residue was resuspended in 4 vol. of acetone, stirred for 5 min and centrifuged at 1000g for 10 min. Acetone washing was repeated twice and the fibre was dried at room temperature giving a brown powder which was stored at -20°C.

Actin was extracted from the powder and purified by the method used for rabbit actin except that after extraction the residue was removed by centrifugation at 10000g for 30 min. The average yield of dried powder was 0.2 g from 1 g of muscle and this yielded 7.0 mg of partially purified actin per g of powder.

Insect actin was purified by gel filtration through Sephadex G-200 (Rees & Young, 1967). Pooled column fractions were concentrated about six times to about 2 mg/ml by ultrafiltration with an Amicon apparatus by using a PM-10 membrane. Concentrated peak fractions were polymerized by dialysing against 0.05 M-KCl-1 mM-MgCl₂-0.3 mM-NaHCO₃, pH 7.0. Pooled fractions 2 and 3 (Fig. 2) contained 17 mg of actin from a total of 40 mg loaded on the column.

Regulatory-protein system

Rabbit tropomyosin was extracted by the method of Bailey (1948) and purified by the method of Hartshorne & Mueller (1969). Rabbit regulatory proteins were extracted by the method of Ebashi & Ebashi (1964) and were salted out at 0°C between 40–60% satd. (NH₄)₂SO₄; troponin was prepared by the method of Ebashi *et al.* (1971). The troponin-tropomyosin complex was reconstituted by mixing tropomyosin and troponin in the ratio 1.0:1.5 by weight.

Insect regulatory proteins were extracted by three methods from the washed myofibrils obtained from 5–20 g of muscle. Sedimented myofibrils were resuspended in 2 vol. of the solution used by Meinrenken (1969) to desensitize fibres, with added dithiothreitol: 0.05 M-KCl, 20 mM-Tris-HCl (pH 8.0) and 2 mM-dithiothreitol. The suspension was dialysed against the solution for 20 h, centrifuged at 10000g for 30 min and the residue was re-extracted for about 10 h with 1 vol. of the solution. The method of Hartshorne & Mueller (1969) was used starting from ether-dried myofibrils. The initial extract in 1 M-KCl, 2 mM-dithiothreitol, pH 7.0, was dialysed for about 16 h against 0.2 M-KCl, pH 6.5–7.0 and centrifuged at 50000g for 30 min to remove paramyosin. The third method was that of Schaub & Perry (1971).

Crude extracts obtained by these methods were fractionated as described by Hartshorne & Mueller

(1969) by precipitating at 40%-satd. $(\text{NH}_4)_2\text{SO}_4$ and between 40–60 or 40–80%-satd. $(\text{NH}_4)_2\text{SO}_4$. The 40–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ -fraction was then precipitated isoelectrically at pH 4.6. Average yields of crude extract were 9.7, 7.0 and 6.5 mg/g of muscle for the methods of Meinrenken (1969), Hartshorne & Mueller (1969) and Schaub & Perry (1971) respectively. The fraction precipitated at 40%-satd. $(\text{NH}_4)_2\text{SO}_4$ was 5.9, 4.0 and 3.5 mg for these extracts and the fraction precipitated at 40–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ was 1.5, 1.3 and 2.3 mg.

Synthetic actomyosin

Myosin and actin were mixed in the ratio 3:1 by weight of myosin/actin, to give a final protein concentration of about 3.0 mg/ml. The mixture was adjusted to contain 0.6 M-KCl, 0.01 M-Hepes [2-(N-2-hydroxyethyl)piperazin-N'-yl]ethanesulphonic acid] (pH 7.0), homogenized in a glass homogenizer and left at 0°C about 30 min. The ionic strength was lowered by dialysing against 0.01 M-Hepes (pH 7.0) for 3–16 h and the precipitated actomyosin redispersed by homogenizing in a glass homogenizer. The initial high-ionic-strength treatment was necessary to ensure binding of insect actin and myosin. Synthetic actomyosins were used immediately after the low-ionic-strength dialysis.

Tropomyosin or regulatory proteins were either added to the actomyosin in the high-ionic-strength solution and precipitated with actomyosin, or they were added to the redispersed actomyosin at low ionic strength; the effect was the same.

Electrophoresis

Sodium dodecyl sulphate electrophoresis was by the method of Weber & Osborn (1969). Gels were 10% (w/v) acrylamide with 0.135% of cross-linker. Samples were dialysed against 1% sodium dodecyl sulphate, 0.01 M-sodium phosphate buffer (pH 7.0) and incubated with 0.1 M-dithiothreitol for 16 h at room temperature before electrophoresis. About 20 µg of protein was loaded on to each gel. Gels were scanned with a densitometer attachment to a Gilford spectrophotometer.

Enzyme assays

ATPase activities were measured by using 0.20–0.50 mg of myosin or actomyosin in 1 ml of medium. For myosin the medium contained 2.5 mM-ATP (potassium salt), 5 or 10 mM- CaCl_2 and 20 mM-Tris-acetate or Tris-maleate buffer; the pH and KCl concentration were as indicated. For myofibrils and actomyosin the medium contained 2.5 mM-ATP (potassium salt), 2.5 mM- MgCl_2 , 25 mM-Tris-HCl buffer, pH 7.6; 2 mM-EGTA (potassium salt) was

added when measuring Ca^{2+} sensitivity. The ATPase activity of synthetic actomyosin over a range of Ca^{2+} concentrations from 1 nM to 10 µM was measured in 2.5 mM-ATP (potassium salt), 2.5 mM- MgCl_2 , 20 mM-Tris-maleate buffer (pH 6.9), 2 mM-EGTA (calcium salt) buffer. For all assays the mixture was equilibrated for 5 min at 25°C and the reaction started by adding 0.05 ml of ATP (potassium salt); P_i released in 5 min was estimated by the method of Fiske & SubbaRow (1925).

The EGTA (calcium salt) buffers were prepared from neutralized stock solutions of 50 mM-EGTA and 48 mM-EGTA (calcium salt) mixed in the proportions calculated to produce the desired Ca^{2+} concentration. The concentration of Ca^{2+} in the medium used for the ATPase assay was calculated by using a version of the program of Perrin & Sayce (1967) modified for use on a PDP8 computer by Dr. R. H. Abbott. The logarithmic stability constant of EGTA (calcium salt) was taken as 10.9 (Holloway & Reilly, 1960).

The potassium salt of ATP was prepared from the disodium salt after treatment with Dowex-50W to remove divalent cations (Seidel & Gergely, 1963).

Protein concentration

Protein concentration was measured by the microbiuret method (Goa, 1953) standardized with Kjeldahl N estimations.

Electron microscopy

Actin filaments in 0.1 M-KCl at a concentration of about 1 mg/ml were put on carbon-coated grids and negatively stained with 1% uranyl acetate. The microscope was an A.E.I. 801A.

Results

Proteins of the myofibril

Washed myofibrils from rabbit psoas muscle, *Lethocerus* and dung beetle flight muscle and *Lethocerus* leg muscle were treated with sodium dodecyl sulphate as described in the Methods section and run on polyacrylamide gels to compare the major proteins of the myofibril (Plate 1). The electrophoresis pattern of the *Lethocerus* and dung beetle myofibrils was similar to that of fly myofibrils (Bullard & Reedy, 1973) except that there was more paramyosin (Bullard *et al.*, 1973) and the protein of about 55000 subunit molecular weight was a single instead of a double band. The dung beetle myofibril had more high-molecular-weight proteins, probably owing to degradation of myosin. A notable difference between the non-fibrillar leg muscle and the fibrillar flight muscle of *Lethocerus* was in the protein of 86000 subunit molecular weight which was absent

from the non-fibrillar muscle. Exhaustive extraction of myosin and actin from flight muscle leaves mainly Z bands; electrophoresis of the residue showed that the proteins of subunit molecular weight 95000 and 86000 made up a large proportion of the total. The 95000-molecular-weight component was probably α -actinin (D. Goll & K. Hammond, unpublished work) and the 86000-molecular-weight protein may also be associated with the Z bands, which are wider in insect fibrillar muscle than in other muscles.

Myosin

The sodium dodecyl sulphate-polyacrylamide-gel electrophoresis patterns of *Lethocerus* and dung-beetle myosins were similar to that of blowfly myosin (Bullard & Reedy, 1973 with a heavy sub-unit of molecular weight about 200000 and two low-molecular-weight subunits (Plates 2a and 2b). Additional proteins in *Lethocerus* myosin were one of subunit molecular weight 107000 and some actin. The component of molecular weight 107000 was probably paramyosin. The light subunits of *Lethocerus* myosin had molecular weights of 17000 ± 500 and 30000 ± 1200 (S.E.M., $n = 10$) as estimated by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. Dung-beetle myosin was almost free of actin but in addition to paramyosin there were proteins of subunit molecular weight 130000 and 80000. The proportion of these proteins increased during the purification procedure whereas the proportion of the heavy subunit decreased, so they were probably derived from the heavy subunit by proteolysis. The subunit molecular weights are about those expected for heavy and light meromyosin from partially digested myosin (Lowey *et al.*, 1969) and they may correspond to these subfragments. The light subunits of dung-beetle myosin had molecular weights of 15000 ± 200 and 27000 ± 1300 (S.E.M., $n = 4$), apparently slightly lower than those of *Lethocerus* myosin. For comparison we measured the molecular weights of the light subunits of myosin from rabbit striated muscle and obtained values of 16000 ± 1700 , 19000 ± 910 and 25000 ± 900 (S.E.M., $n = 6$) similar to those of Weeds & Lowey (1971). Therefore the molecular weights, at least of the heaviest light chains, of insect myosins appear to be different from those of rabbit striated-muscle myosin. However, sodium dodecyl sulphate-polyacrylamide-gel electrophoresis can give incorrect values for the molecular weights of myosin light chains (Weeds & Lowey, 1971).

The ATPase activity of the insect myosins was activated by Ca^{2+} and inhibited by Mg^{2+} , in agreement with Maruyama (1959). In the presence of 50 mM-KCl, at pH 7.6 with 5 mM- CaCl_2 the average ATPase activity was $0.16 \mu\text{mol of } \text{P}_i/\text{min per mg}$ for *Lethocerus* and 0.13 for dung beetle; in the same medium with 2.5 mM- MgCl_2 instead of CaCl_2 the

activities were 0.017 for *Lethocerus* and 0.003 for dung beetle.

The effect of ionic strength and pH on the ATPase activity of the insect myosins is shown in Fig. 1, together with values for rabbit myosin. The conditions of the assay were similar to those used by Bárány & Bárány (1966) so the ATPase activity of the insect myosins can be compared with the values obtained for scallop myosin by these authors. The ionic strength dependence of the insect myosin ATPase activity was similar to that of rabbit myosin with the highest activity below 0.1 M-KCl, and differed from

Fig. 1. Effect of ionic strength and pH on the ATPase activity of insect and rabbit myosin

The assay medium contained the myosin specified, 2.5 mM-ATP, 10 mM- CaCl_2 and 20 mM-Tris-acetate. (a) The effect of KCl concentration at pH 6.5; (b) the effect of pH with 0.5 M-KCl. $\circ$, Rabbit myosin (0.19 mg); $\bullet$, dung-beetle myosin (0.25 mg) prepared in the presence of 1 mM-dithiothreitol; Δ , *Lethocerus* myosin (0.25 mg) prepared in the presence of 14 mM-2-mercaptoethanol.

EXPLANATION OF PLATE I

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of myofibrils

(a) Rabbit back muscle, (b) *Lethocerus* leg muscle, (c) *Lethocerus* flight muscle, (d) dung-beetle flight muscle. For details see the text.

EXPLANATION OF PLATE 2

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of insect myosin and actin preparations

Myosin re-precipitated once at 0.03 M-KCl: (a) *Lethocerus* myosin, (b) dung-beetle myosin. Actin polymerized three times: (c) dung-beetle actin before column chromatography, (d) the same actin eluted in the pooled fractions 1 of Sephadex G-200 column (see Fig. 2), (e) actin in pooled fractions 2, (f) actin in pooled fractions 3, (g) *Lethocerus* actin before column chromatography, (h) the same actin eluted from Sephadex G-200 column.

EXPLANATION OF PLATE 3

(a) *Electron micrographs of impure dung-beetle actin and (b) electrophoresis of impure dung-beetle actin*

Actin was polymerized once in 0.1 M-KCl, pH 6.3. (a) Negatively stained actin filaments. The arrows show protein bound with a periodicity of about 40 nm. The magnification of both micrographs is $\times 162\,000$. (b) Sodium dodecyl sulphate-polyacrylamide gel of actin after being polymerized once.

EXPLANATION OF PLATE 4

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of Lethocerus regulatory-protein preparation

Protein was extracted with Meinrenken's (1969) solution as described in the text. (a) Fraction precipitated at 40%-satd. $(\text{NH}_4)_2\text{SO}_4$, (b) fraction precipitated from 40-80%-satd. $(\text{NH}_4)_2\text{SO}_4$, (c) supernatant obtained by adjusting 40-80%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction to pH 4.6 and removing the precipitate.

scallop, where the maximum activity is at 0.2M-KCl. The pH dependence, however, was similar for insect and scallop myosin: the activity decreased above pH 8.5, whereas the activity of myosin from rabbit fast muscle increased above pH 8.5. Maruyama (1954) found a similar pH dependence for the ATPase activity of housefly actomyosin. The loss of ATPase activity at alkaline pH is also characteristic of myosins from vertebrate slow muscle (Sreter *et al.*, 1966; Bárány & Close, 1971). The high ATPase activity of *Lethocerus* myosin compared with dung-beetle myosin (Fig. 1) may be attributed to the 2-mercaptoethanol used in preparing that sample of *Lethocerus* myosin; this reagent activates the ATPase of myosin from vertebrate striated muscle (Trayer & Perry, 1966).

Actin

Actin purified by polymerizing and depolymerizing three times, including once in 0.6M-KCl, was contaminated with various amounts of protein of subunit molecular weight 55000. Gel filtration of dung beetle actin on Sephadex G-200 gave the elution pattern shown in Fig. 2 and the fractions were pooled as shown. Most of the contaminating proteins of molecular weight 55000 and higher, if present, were eluted in the pooled fractions number 1 (Fig. 2); pooled fractions 2 and 3 contained relatively pure actin. Sodium dodecyl sulphate-polyacrylamide gels

Fig. 2. Chromatography of dung-beetle actin on Sephadex G-200

Sephadex G-200 was equilibrated with 0.5mM-ATP, 0.5mM-2-mercaptoethanol, 0.2mM- CaCl_2 , pH 7.5 at 4°C; 40mg of actin in 10ml of the same solvent was loaded on the column (2cm×35cm). The flow rate was 5ml/24min. The fractions were pooled in groups 1, 2 and 3, as indicated by the bars.

of column fractions are shown in Plate 2. The purification of *Lethocerus* actin by gel filtration was less successful and after elution from the column the actin was still associated with some protein of molecular weight 55000 (Plate 2). Electron microscopy of the pooled fractions from the column, in 0.1M-KCl, showed that pooled fraction 1 (Fig. 2) was amorphous aggregated material; pooled fractions 2 and 3 had long filaments but these appeared less rigid than filaments of actin polymerized before running through the column.

Periodic binding of protein to actin filaments

Electron micrographs of impure actin from dung beetle showed filaments with protein bound at intervals of about 40nm (Plate 3). The troponin of vertebrate striated muscle binds to actin filaments containing tropomyosin, at about the same periodicity (Ohtsuki *et al.*, 1967; Hanson, 1972; Spudich *et al.*, 1972). However, sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of the impure insect actin (Plate 3) showed that there was protein of molecular weight 55000 as well as tropomyosin and troponin, so it is not possible to tell which protein is bound periodically along the actin.

Regulatory proteins

Unfractionated extracts of regulatory proteins from flight muscle, obtained by the methods described, had an inhibitory effect on the ATPase of desensitized or synthetic actomyosin from rabbit; the inhibition was usually greater in the absence of Ca^{2+} . This is in agreement with the results of Meinrenken (1969), which show that extracts of insect regulatory proteins inhibit the ATPase of rabbit synthetic actomyosin even in the presence of Ca^{2+} . Table 1 shows the effect of typical extracts and fractions. The proteins precipitating at 40%-satd. $(\text{NH}_4)_2\text{SO}_4$ had an inhibitory effect that was usually independent of Ca^{2+} concentration, whereas those precipitating between 40-80% saturation had inhibitory activity that was greater in the absence of Ca^{2+} . The extent of inhibition and the sensitivity to Ca^{2+} varied from preparation to preparation.

Sodium dodecyl sulphate - polyacrylamide-gel electrophoresis showed that the initial extracts contained high-molecular-weight proteins, in addition to tropomyosin and troponin components. Extracts made from an ether-dried powder by the method of Hartshorne & Mueller (1969) were more degraded, having more proteins of subunit molecular weight 14000 or less (Drabikowski *et al.*, 1971), than extracts made by other methods; these extracts were often very active but occasionally had no activity at all.

The 40-80% satd. $(\text{NH}_4)_2\text{SO}_4$ fraction contained tropomyosin and proteins of subunit molecular

Table 1. *Effect of insect regulatory proteins on the ATPase activity of desensitized and synthetic actomyosin*

About 0.20mg of actomyosin and an equal amount of regulatory protein were assayed in a medium containing 2.5mM-ATP, 2.5mM-MgCl₂, 25mM-Tris-HCl (pH 7.6) and 2mM-EGTA where indicated. Regulatory protein was prepared from *Lethocerus* by the method of Meinrenken (1969) in Expt. 1 and Hartshorne & Mueller (1969) in Expt. 2; from dung beetle by the method of Schaub & Perry (1971) in Expts. 3 and 4. Fractions were tested on rabbit desensitized actomyosin in Expts. 1 and 3, on rabbit synthetic actomyosin in Expt. 2 and on synthetic actomyosin with *Lethocerus* myosin plus rabbit actin plus 0.10mg of rabbit tropomyosin, in Expt. 4. % inhibition = $100 \times [1 - (\text{ATPase activity with regulatory protein} / \text{ATPase activity without regulatory protein})]$.

Regulatory protein fraction		% inhibition of ATPase activity	
		No EGTA	EGTA
Expt. 1	Crude extract	70	50
	40%-satd. (NH ₄) ₂ SO ₄	45	40
	40-80%-satd. (NH ₄) ₂ SO ₄	0	57
Expt. 2	Crude extract	25	76
	40%-satd. (NH ₄) ₂ SO ₄	32	23
	40-80%-satd. (NH ₄) ₂ SO ₄	0	65
	Insect troponin plus rabbit tropomyosin	0	65
Expt. 3	Crude extract	50	66
	40%-satd. (NH ₄) ₂ SO ₄	48	50
	40-80%-satd. (NH ₄) ₂ SO ₄	8	61
Expt. 4	40-80%-satd. (NH ₄) ₂ SO ₄	6	60

weight 18000, 27000 and 30000. Since this fraction produced Ca²⁺-sensitive inhibition (Table 1) it must contain the components of troponin. When the fraction was brought to pH 4.6, tropomyosin was precipitated, but the yield of troponin in the supernatant was low and sometimes the proteins were degraded. The troponin, when combined with rabbit tropomyosin, had inhibitory activity with strong Ca²⁺ sensitivity (Table 1). Plate 4 shows sodium dodecyl sulphate-polyacrylamide gels of regulatory protein extracted with Meinrenken's (1969) solution that gave the clearest separation of troponin.

A large part of the component of molecular weight 27000 was in the 40%-satd.-(NH₄)₂SO₄ fraction and since the activity of this fraction was inhibitory, with little Ca²⁺-sensitizing activity, the component is probably analogous to the inhibitory factor of rabbit troponin. Similarly, a fraction extracted from vertebrate striated muscle and precipitating at 30%-satd. (NH₄)₂SO₄ has inhibitory activity that is independent of Ca²⁺ concentration (Perry *et al.*, 1966; Hartshorne *et al.*, 1967) and this is probably due to the inhibitory factor of troponin (Schaub & Perry, 1969).

Only those extracts with the component of molecular weight 18000 had Ca²⁺-sensitizing activity, so this is probably analogous to the Ca²⁺-sensitizing factor of rabbit troponin. The function of the component of molecular weight 30000 is uncertain. Active troponin preparations often had very little of the component of molecular weight 27000 after frac-

tionation (Plate 4) so the protein of molecular weight 30000 may also be an inhibitory factor. Alternatively it may be analogous to the 37000-molecular-weight component of rabbit troponin; proteolysis of rabbit troponin degrades the 37000-molecular weight component to a protein of molecular weight 30000 (Drabikowski *et al.*, 1973; Dabrowska *et al.*, 1973).

Restoration of Ca²⁺ sensitivity to insect myofibrils

The effectiveness of troponin-containing extracts from rabbit and insect muscle in producing Ca²⁺-sensitive inhibition in desensitized *Lethocerus* myofibrils was compared. Fig. 3 shows that the 40-60%-satd.-(NH₄)₂SO₄ fractions of insect and rabbit extracts were about equally inhibitory in the absence of Ca²⁺, but in the presence of Ca²⁺ the insect extract was still somewhat inhibitory whereas the rabbit extract produced slight activation. The effect of both extracts on insect myofibrils was similar to their effect on rabbit actomyosin (Table 1 and Schaub & Perry, 1969).

Synthetic actomyosins

Synthetic actomyosins were made from *Lethocerus* and rabbit proteins in four combinations of myosin and actin. Fig. 4 shows the dependence of the ATPase activity of these actomyosins on Ca²⁺ concentration from 1 nM to 10 μ M. The actomyosin ATPase activity was measured without any additions, with added rabbit tropomyosin, with added rabbit tro-

Fig. 3. Effect of *Lethocerus* and rabbit regulatory protein on the ATPase activity of desensitized *Lethocerus* myofibrils

The assay medium contained 0.37 mg of desensitized *Lethocerus* myofibrils, 2.5 mM-ATP, 2.5 mM-MgCl₂, 25 mM-Tris-HCl (pH 7.6). (a) *Lethocerus* 40-60%-satd. (NH₄)₂SO₄ fraction of protein extracted with Meinrenken's (1969) solution; (b) rabbit, 40-60%-satd.-(NH₄)₂SO₄ fraction of extract prepared by method of Ebashi & Ebashi (1964) ○, No EGTA; ●, with 2 mM-EGTA.

ponin-tropomyosin complex and with our most active preparation of insect regulatory protein. The ATPase activity of the actomyosins containing insect myosin, without additions, was low compared with that of actomyosins containing rabbit myosin and was insensitive to Ca²⁺ concentration. The activity was increased on adding rabbit tropomyosin, but did not become Ca²⁺ dependent (Bullard *et al.*, 1972). The effect of tropomyosin is shown for *Lethocerus* myosin and rabbit actin in Fig. 4(b), where the actomyosin ATPase activity was increased 2.2-2.3

times by tropomyosin; activation occurred to the same extent with insect myosin and insect actin, although it was not measured for the particular preparation of Fig. 4(d). The greatest activation was obtained with dung-beetle myosin and rabbit actin, where the ATPase activity in the presence of tropomyosin was 3.2 times that of the actomyosin alone. Adding rabbit troponin-tropomyosin complex or insect regulatory protein made the ATPase activity of the actomyosins sensitive to Ca²⁺. With 1 nM-Ca²⁺ the ATPase activity was similar for all actomyosins, and where there was insect myosin this was about the same value as without additions. With 10 μM-Ca²⁺ the ATPase activity was greater for actomyosins containing rabbit actin than insect actin. There was no significant difference in the Ca²⁺ concentration required for half-maximal activation of the different actomyosins.

The Ca²⁺ sensitivities of synthetic actomyosins with rabbit and insect regulatory protein are given in the legend of Fig. 4. Combinations with rabbit actin and rabbit or insect myosin had the same fairly high Ca²⁺ sensitivity with rabbit troponin-tropomyosin complex, and this was the same as that for rabbit actomyosin with insect regulatory protein. Actomyosins with insect actin had a somewhat lower Ca²⁺ sensitivity that was about the same with either rabbit or insect myosin. The lower ATPase activity at high Ca²⁺ concentration for actomyosins with insect actin, compared with actomyosins with rabbit actin, may be due to column purification of the insect actin, which appears to make it more flexible, or to 55000-molecular-weight protein remaining with the actin. However the activity of ATPase and the Ca²⁺ sensitivity of all the actomyosins were within the range of values found for rabbit actomyosin and troponin-tropomyosin complex.

Discussion

The isolation of myosin, actin and troponin from insect fibrillar muscle has revealed some differences between the insect proteins and those of vertebrate striated muscle. Although the myosins from both kinds of muscle have about the same molecular weight (Bullard & Reedy, 1973), the light-subunit compositions differ. As with myosins from slow and fast vertebrate muscle (Sarkar *et al.*, 1971), it is not possible to tell whether the difference in the pH dependence of the ATPase activity is due to the light subunits or to a difference in the heavy subunits of the myosin molecule. The low ATPase activity of actomyosins containing insect myosin and their activation by tropomyosin may be due to some property of the myosin that prevents it interacting with actin in the absence of tropomyosin. The same phenomenon has been observed in *Limulus* myosin (Lehman & Szent-Györgyi, 1972). The paramyosin in our

Fig. 4. Ca^{2+} dependence of the ATPase activity of synthetic actomyosins

The assay medium contained 0.19mg of actomyosin, 2.5mM-ATP, 2.5mM-MgCl₂, 20mM-Tris-maleate (pH 6.9), 2mM-EGTA (calcium salt) buffer and 0.09mg of tropomyosin or 0.19mg of regulatory protein where indicated. (a) Rabbit actin and myosin, (b) rabbit actin and *Lethocerus* myosin, (c) *Lethocerus* actin and rabbit myosin, (d) *Lethocerus* actin and myosin. ○, No additions; △, with rabbit tropomyosin, ●, with rabbit troponin-tropomyosin complex; □, with *Lethocerus* 40–80%-satd.-(NH₄)₂SO₄ fraction of extract prepared by the method of Hartshorne & Mueller (1969). Ca^{2+} sensitivity with rabbit troponin-tropomyosin complex is 68% for (a), 67% for (b), 59% for (c), 59% for (d) and 68% for (a) with *Lethocerus* regulatory protein. Ca^{2+} sensitivity = $100 \times [1 - (\text{ATPase activity at } 1 \text{ nM-}\text{Ca}^{2+} / \text{ATPase activity at } 10 \mu\text{M-}\text{Ca}^{2+})]$.

myosin preparations does not cause the low actomyosin ATPase activity, or the activation by tropomyosin since the same behaviour is found with paramyosin-free insect myosin from dung beetle or *Lethocerus* (K. Hammond, unpublished work). The activation of the ATPase by tropomyosin is the most striking difference between the insect and vertebrate contractile systems. Stretch-activation in insects may possibly involve a mechanical effect on the interaction between actin and tropomyosin that increases the affinity of myosin and actin.

The effect of the troponin-tropomyosin complex on desensitized insect myofibrils (Fig. 3) is to inhibit the ATPase activity in the absence of Ca^{2+} , relative to the value without regulatory proteins. This is in contrast with the effect on synthetic actomyosins containing insect myosin (Figs. 4b and d) where the ATPase is activated in the presence of Ca^{2+} , relative to the value without regulatory proteins. Desensitizing flight muscle myofibrils and actomyosin some-

times decreases the ATPase activity relative to that of sensitive preparations at high Ca^{2+} concentration: Maruyama *et al.* (1968) observed a decrease for actomyosin but not myofibrils, after desensitizing by trypsin digestion, and Meinrenken (1969) found a decrease after desensitizing myofibrils by washing. The activity of the ATPase in desensitized preparations probably depends on how much tropomyosin was removed during the desensitizing process; for the myofibrils used in Fig. 3 some tropomyosin was seen in the sodium dodecyl sulphate-polyacrylamide gels after desensitizing.

Insect actin is similar to rabbit actin in monomer molecular weight and polymerizing properties (Maruyama, 1959) and in its capacity to bind protein with a periodicity of 40nm in the presence of tropomyosin, but it differs in being associated with a protein of subunit molecular weight 55000. This protein is not present in extracts containing the troponin-tropomyosin complex or in active troponin

preparations, so is unlikely to be a troponin component. The subunit molecular weight, solubility and polymerizing property are characteristic of tubulin (Shelanski & Taylor, 1968; Stephens, 1968; Weisenberg *et al.*, 1968) but we have no further evidence for the identity of this protein.

Extracts of regulatory protein from insect muscle apparently have an excess of inhibitory factor over Ca^{2+} -sensitizing factor. We cannot determine from the methods used here whether this is due to a low proportion of Ca^{2+} -sensitizing factor in the myofibril or, alternatively, to preferential extraction of inhibitory factor or denaturation of Ca^{2+} -sensitizing factor. Once fractionated to remove excess of inhibitory factor, insect troponin produces as great a sensitivity to Ca^{2+} as rabbit troponin.

The experiments with synthetic actomyosins have shown no significant difference between insect and rabbit proteins in the Ca^{2+} -sensitivity of the ATPase activity with added troponin-tropomyosin complex. The Ca^{2+} -sensitivity of the synthetic actomyosins with troponin and tropomyosin is about the same as that observed by Maruyama *et al.* (1968) for myofibrils from fibrillar muscle. Although these authors found less Ca^{2+} -sensitivity in the actomyosin than in the myofibrils from fibrillar muscle, both their results with intact myofibrils, and ours with synthetic actomyosins, show that fibrillar muscle has about the same Ca^{2+} -sensitivity as vertebrate striated muscle. The property of stretch-activation in insect muscles is not, therefore, associated with unusually low Ca^{2+} sensitivity of the actomyosin.

We have found regulatory proteins similar to those associated with thin filaments in vertebrates but the myosin, isolated by the methods used here, apparently lacked the Ca^{2+} -sensitive components suggested by the experiments of Lehman *et al.* (1973). However the troponin system alone was sufficient to produce Ca^{2+} sensitivity comparable with that of actomyosin from vertebrate striated muscle.

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