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Regulatory Proteins of Lobster Striated Muscle†

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ABSTRACT: The regulatory proteins of lobster muscles consist of tropomyosin and of troponin. Troponin contains a 17,000 chain weight component, two closely related components of about 30,000 and a 52,000 chain weight component. In addition to troponin, tropomyosin is required for the inhibition of the magnesium activated actomyosin ATPase activity in the absence of calcium and for the reversal of this inhibition by calcium. Lobster tropomyosin interacts with rabbit actin and lobster troponin interacts with rabbit tropomyosin. The 30,000 doublet component corresponds to the troponin-I of rabbit and inhibits the ATPase activity of actomyosin both in the presence and in the absence of calcium. The 17,000 component corresponds to the troponin-C of rabbit; it binds calcium and reverses the inhibition of the ATPase activity by troponin-I in the presence of calcium. No more than 1 mol of calcium is bound by a mole of troponin-C or by troponin. The 52,000 component interacts with tropomyosin and has been tentatively identified as troponin-T; however, it has not been demonstrated as yet that this component had a role in the regulation of lobster actomyosin.

The mechanism of troponin action in the calcium regulation of contraction has been studied in detail only in vertebrate striated muscles (*cf.* Ebashi and Endo, 1968; Weber and Murray, 1973). Rabbit troponin was shown to contain three different subunits (Greaser and Gergely, 1971). Troponin-I (TN-I)[1] inhibits the actin activated ATPase both in the presence and in the absence of calcium (Perry *et al.*, 1972; Ebashi *et al.*, 1972; Greaser *et al.*, 1972; Hartshorne and Dreizen, 1972). Troponin-C (TN-C) is the calcium binding protein, and in the presence of calcium it removes the TN-I induced inhibiton of the ATPase (Hartshorne and Mueller, 1968). Troponin-T (TN-T) binds to tropomyosin, and it is required together with tropomyosin for calcium regulated control (Greaser and Gergely, 1971). The components of chicken troponin, although less extensively studied, are similar to rabbit (Hitchcock *et al.*, 1973).

The presence of troponin has also been shown in insect muscles (Maruyama *et al.*, 1968; Meinrenken, 1969; Bullard *et al.*, 1973), and in *Limulus* (Lehman *et al.*, 1972). An actin-linked regulation requiring tropomyosin has been demonstrated in a number of invertebrate muscles, and it may coexist with the myosin-linked regulatory system, as in insects and annelids, or alternatively it functions alone as in most decapod muscles (Lehman *et al.*, 1972, 1974).

These studies also indicate that invertebrate troponin is a multicomponent protein system (Lehman *et al.*, 1972; Bul-

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[1] Abbreviations used are: TN, troponin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; SDS, sodium dodecyl sulfate.

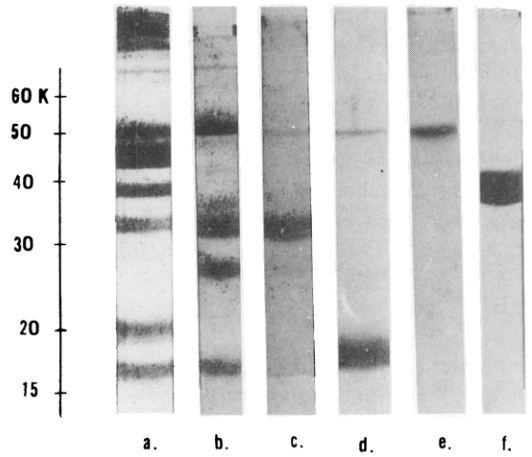

FIGURE 1: Sodium dodecyl sulfate acrylamide gel electrophoresis of lobster proteins: (a) natural actomyosin; (b) troponin; (c) TN-I; (d) TN-C; (e) TN-T; (f) tropomyosin. 12% gels stained with Coomassie Brilliant Blue. Note presence of 27,000-dalton component in troponin.

lard *et al.*, 1973); however, the components of these invertebrate troponins have not been isolated and their activity has not yet been tested individually.

In this study we focused our attention on the isolation and characterization of the regulatory components of a crustacean muscle. The function of the subunits of troponin and of the obligatory role of tropomyosin in actin-linked regulation were studied. We were trying to determine the basic similarities and differences between vertebrate and crustacean troponins. Homologous features of the actin-linked regulation in animals which are widely separated phylogenetically would signify that these features may be essential for regulation and were conserved. Similarly, differences may point out those properties which have undergone adaptive changes without effecting the regulatory function. Lobster muscle was chosen because in crustacean muscle as in rabbit and chicken muscle actin-linked regulation is the only functioning regulatory system present. Moreover, lobster is a good source of large amounts of muscle, and troponin and its subunits can be readily prepared for comparison with their vertebrate counterparts.

Experimental Section

Preparations

The solutions used for protein preparations contained 0.1 mM dithiothreitol and their temperature was maintained at 0–4° throughout; ammonium sulfate fractionation, however, was conducted at room temperature.

Myofibrils. The tail muscle of freshly killed lobster, *Homarus americanus,* was homogenized in a Sorvall Omnimixer in 0.04 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 5 mM sodium phosphate buffer at pH 7.4, and dithiothreitol. The myofibrils were collected by a 10-min centrifugation at 16,000*g* in a Sorvall centrifuge. The fibrils were washed twice with the same solution, then rehomogenized and suspended in 0.04 M NaCl and 5 mM phosphate (pH 7.4). The myofibrils were frequently stored in 50% glycerol containing 0.04 M NaCl. Glycerol was removed by centrifuging a suspension that was diluted fivefold with 0.04 M NaCl. For the preparation of relaxed myofibrils, the muscles were placed *in situ* into a wash solution, containing 50% glycerol for a day prior to homogenization.

Natural Actomyosin. Actomyosin was extracted from freshly prepared washed myofibrils by a few minutes gentle stirring in 0.6 M NaCl, 5 mM phosphate (pH 7.4,) and 5 mM ATP. The insoluble residue was removed by 10-min centrifugation at 30,000*g*. The supernatant solution was diluted with 12 volumes of 5 mM phosphate (pH 6.5), centrifuged at 16,000*g* and resuspended in 0.04 M NaCl and 5 mM phosphate (pH 7.4) (Figure 1a).

Desensitized Myofibrils and Actomyosin. The actomyosin or myofibril preparations were dialyzed four to six times against 10 volumes of water for a period of about 12 hr each. The desensitized myofibrils or actomyosin were collected by 10-min centrifugation at 30,000*g*. These preparations were not calcium sensitive. The supernatant was used for the preparation of the regulatory proteins.

Regulatory Proteins. Ammonium sulfate (21 g to 100 ml) was added to the supernatant solution obtained from desensitized preparations, in 0.2 M NaCl and 5 mM Tris (pH 7.0). Precipitate was removed by centrifugation at 30,000*g*. The regulatory proteins were precipitated by further addition of ammonium sulfate (24 g/100 ml), collected by centrifugation, dissolved in 0.6 M NaCl, dialyzed against the same solution, and clarified by a 10-min centrifugation at 30,000*g*. The solution was dialyzed against 0.04 M NaCl–5 mM phosphate (pH 7.4) and clarified again by 10-min centrifugation at 30,000*g* (Figure 2a).

Tropomyosin. The pH of the regulatory proteins in 1.0 M NaCl and 10 mM sodium citrate was adjusted to 4.7 with 0.5 M HCl. Tropomyosin was precipitated and collected by 10-min centrifugation at 30,000*g*. The supernatant solution contained troponin. The precipitate was taken up in 0.6 M NaCl–10 mM Tris (pH 8.0), dialyzed against the same solution, and clarified by centrifugation. Preparations were frequently stored at −20°. Tropomyosin was further purified by precipitation from a 0.05 M Tris (pH 8.0) solution by dialysis against 0.02 M $MgCl_2$–0.05 M Tris (pH 8.0). The collected paracrystals were dissolved in 0.6 M NaCl, and tropomyosin was precipitated with 10 mM sodium acetate at pH 5.2 in the presence of 0.12 M ammonium sulfate. The magnesium and acid precipitation steps were repeated a second time (Figure 1f).

Troponin. The supernatant remaining after the removal of tropomyosin at pH 4.7 was neutralized and dialyzed against water (Figure 1b). Small amounts of precipitate were removed by centrifugation and the supernatant was concentrated by ammonium sulfate precipitation (47 g/100 ml) in 0.2 M NaCl–10 mM Tris (pH 8.0). The precipitate was taken up and dialyzed against 6 M urea (freshly prepared)–0.02 M sodium citrate (pH 6.2). The solution was clarified by a 1-hr centrifugation at 100,000*g* and chromatographed on a SE-Sephadex C-50 column (3.8 × 15 cm) which was equilibrated with the same buffer. The fractions in the void volume and those eluted with 0.1 M NaCl buffer were combined and contained TN-T and TN-C. The TN-I bound onto the column and was eluted with 0.4 M NaCl in the equilibrating buffer.

Troponin-T and Troponin-C. The combined void volume and 0.1 M NaCl fractions which were eluted from the SE-Sephadex column were dialyzed to remove urea and concentrated by precipitation with ammonium sulfate (47 g/100 ml). TN-T and TN-C were resuspended in 0.04 M NaCl and 5 mM phosphate (pH 7.4) and separated by exhaustive dialysis against 0.04 M NaCl, 5 mM phosphate (pH 7.4), and 1 mM ATP. The precipitate consisted of TN-T; the supernatant solution contained enriched TN-C (Figure 1d). A second dialysis yielded TN-C essentially free

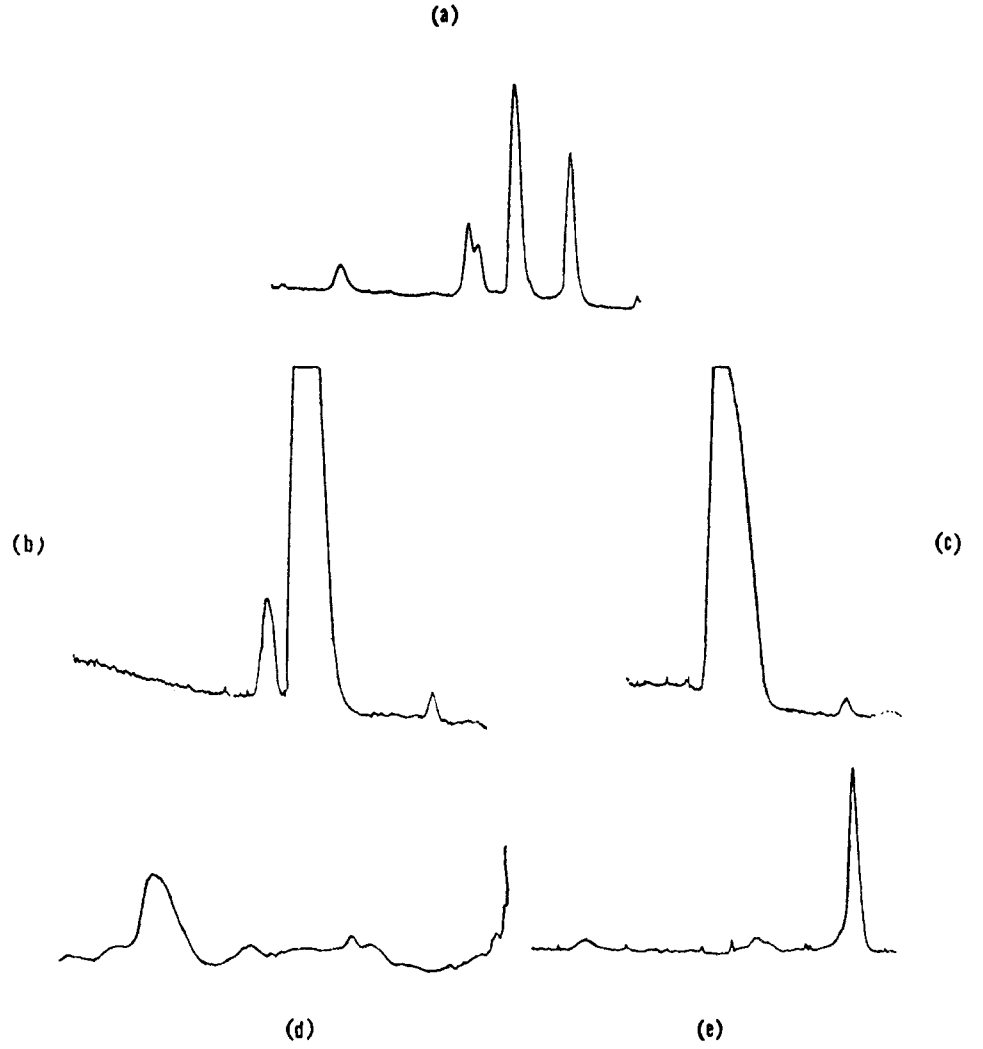

FIGURE 2: Densitometry of sodium dodecyl sulfate acrylamide gels: (a) regulatory proteins; (b) TN-I; (c) TN-I purified by 10-min incubation at 100°; (d) TN-C; (e) TN-T. 12% gels stained with Fast Green. Proteins migrated from right to left. Note that heat treatment removes 27,000-dalton impurity from TN-I. Poor base line indicates higher molecular weight impurities in TN-C preparation. The 43,000-dalton component in natural actomyosin is actin; the 19,000-dalton component is a myosin light chain (Lb 1); the 17,000-dalton component is a combination of the other myosin light chain (Lb 2) and TN-C.

of TN-T (Figure 2d). TN-T was solubilized in 4 M urea and clarified (Figures 1e and 2e); however, a significant portion of the protein did not dissolve.

Troponin-I. The fractions eluted by 0.4 M NaCl–urea–citrate buffer from the SE-Sephadex column were dialyzed exhaustively to remove urea and concentrated by ammonium sulfate precipitation (47 g/100 ml) (Figures 1c and 2b). The preparation contained an impurity having a chain weight of 27,000. This component could be removed either by a 7.5% trichloroacetic acid precipitation or by 10-min boiling followed by dialysis against 0.04 M NaCl–5 mM phosphate (pH 7.4) and clarification for 10 min at 30,000*g*. The supernatant consisted predominantly of TN-I (Figure 2c). Most of the experiments, however, were performed with TN-I preparations which were not subjected to trichloroacetic acid or boiling.

Rabbit Myosin. Myosin was prepared from the back and leg muscles by extraction with 0.3 M KCl–0.15 M phosphate buffer (pH 6.5) for 10 min (Szent-Gyorgyi, 1951; Mommaerts and Parrish, 1951). After a sequence of steps including clarification at 0.6 M NaCl, removal of actomyosin at 0.29 M NaCl, and precipitation at 0.05 M NaCl had been carried out twice, the myosin was stored in 50% glycerol containing 0.6 M NaCl–5 mM phosphate (pH 7.4) at −20°.

Rabbit Actin. Preparations routinely showing a single band in SDS acrylamide gel electrophoresis were obtained by the following procedure (Straub, 1942; Mommaerts, 1952; Drabikowski and Gergely, 1964): The muscle residue following myosin extraction was collected on cheesecloth and washed for 30 min in 5 volumes of 0.4% $NaHCO_3$–0.1 mM $CaCl_2$. The muscle residue was collected on cheesecloth and then resuspended and stirred for 10 min in one volume of cold 10 mM $NaHCO_3$, 10 mM Na_2CO_3, and 0.1 mM $CaCl_2$. Ten volumes of room temperature water were added to the suspension and the liquid was quickly removed by filtration through cheesecloth. The residue was dehydrated and the fat was removed by repeated acetone treatments. The powder was stored at −20°. Actin was extracted from the powder with cold CO_2-free water and, following 60 min clarifying centrifugation at 100,000*g*, it was polymerized at room temperature with 10 mM NaCl and 0.7 mM $MgCl_2$. Polymerized actin was collected by 4 hr centrifugation at 100,000*g* and resuspended in 0.05 M NaCl. The presence of $CaCl_2$ in the wash solutions and the use of low concentrations of $MgCl_2$ and NaCl for polymer-

ization are important to obtain an actin free of troponin and tropomyosin.

Actomyosin was prepared weekly by mixing fresh F-actin (prepared from the stored acetone powder) with myosin, which had been dialyzed against 0.6 M NaCl to remove the glycerol, in a ratio of 1:3-4 (w/w).

Procedures

Calcium Binding. Samples were dialyzed twice for 12 hr against 100 volumes of solutions containing 0.04 M NaCl, 10 mM imidazole (pH 7.0), 1 mM $MgCl_2$, and an appropriate mixture of 0.04 mM ^{45}CaEGTA and EGTA to obtain the desired free calcium concentrations using a dissociation constant of 1.9×10^{-7} for CaEGTA at pH 7.0. $MgCl_2$ reduced nonspecific divalent cation binding. At pH 7.0 these additional ions do not affect the dissociation constant of CaEGTA significantly (Regenstein, 1972); 0.5 ml of the protein solution and dialyzing liquid were counted in a Beckman LS-255 liquid scintillation counter using a scintillation cocktail consisting of 5 ml of toluene, 2,4-diphenyloxazole, and 1,4-bis[2-(5-phenyloxazolyl)]benzene and 1 ml of Biosolve. A solution, free of protein, containing nonradioactive buffer, was also dialyzed against the ^{45}CaEGTA buffers to check that equilibrium had been achieved. Protein concentrations were sufficiently high that the counts in the protein solution exceeded the counts in the dialyzing liquid by 10% or more, provided a calcium binding protein was present.

ATPase Measurements. ATPase activity was followed by measuring the rate of proton liberation at pH 7.6 using a Radiometer pH-Stat. The magnesium activated ATPase activity of actomyosin was measured in the absence of calcium in a solution of 0.03 M NaCl, 1 mM $MgCl_2$, and 0.1 mM EGTA ($Mg\text{-}ATPase_{EGTA}$). The reaction was started with 0.5 mM ATP, and the ATPase activity in the absence of calcium was measured for about 3-5 min. The measurement was continued for an additional 3-5 min following the introduction of 0.2 mM $CaCl_2$ and the Mg-ATPase activity in the presence of calcium ($Mg\text{-}ATPase_{Ca}$) was obtained from the new slope. The pH and magnesium dependence of the dissociation of CaEGTA was corrected for; the correction amounted to nearly one pCa unit from pH 7.0 to pH 7.6 (Regenstein, 1972).

Protein Determinations. The amount of protein was determined by the procedure of Lowry *et al.* (1951) using bovine serum albumin as a standard. Bovine serum albumin was standardized by Kjeldahl nitrogen analysis.

Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed by the method of Weber and Osborn (1969). Gels of 12% acrylamide were used in order to ensure the separation of components in the 10,000-50,000 region. The *N,N'*-methylenebisacrylamide concentration was also increased by a factor of 1.2 from that used with the normal 10% gels. Gels were stained either with fresh Coomassie Brilliant Blue (0.25%) or Fast Green (0.5%) in 50% methanol-10% acetic acid at 40°.

Densitometry. Fast Green stained gels were placed in a special holder to give an optically flat surface and scanned on the Joyce-Loebel double beam recording densitometer MK III C with a 0-1.5 optical density wedge (*cf.* Regenstein, 1972).

Amino Acid Analysis. Analysis was performed on a Beckman Model 120B analyzer on protein samples hydrolyzed for 24 hr at 110° in reagent grade concentrated HCl, diluted 1:1 with water.

Electron Microscopy. A Philips EM 300 was used at 80 kV to examine the tropomyosin paracrystals that were negatively stained with uranyl acetate.

Chemicals. Ammonium sulfate and urea (enzyme grades) were preparations of Schwarz/Mann, $^{45}CaCl_2$ was purchased from New England Nuclear. Dithiothreitol, ATP, and Tris were purchased from Sigma Chemical Co., and the EGTA was obtained from K & K Laboratories, Plainview, N.J. The technical grade sodium dodecyl sulfate from Matheson Coleman and Bell was used without further purification. Biosolve was obtained from Beckman Instruments, and 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]benzene were from Packard Instruments. All otherwise unspecified chemicals were reagent grade from Fisher Scientific Co., Eastman Kodak Co., or J. T. Baker.

Results

General Features. Washed myofibrils and actomyosin preparations of lobster require low concentrations of calcium for the magnesium-activated ATPase activity. In the absence of calcium, ATPase activity is lowered several-fold; the per cent calcium sensitivity[2] may vary from 60 to nearly 100%. Contracted myofibrils, however, frequently do not show calcium sensitivity unless they are redissolved in high salt in the presence of ATP. ATPase studies were restricted to rest length or slightly stretched myofibrils obtained by *in situ* glycerination.

The presence of an actin-linked regulation in lobster muscles is indicated by the competitive actin binding test (Lehman *et al.*, 1972). This test is based on the observation that troponin-tropomyosin reduces the affinity of actin to myosin in the absence of calcium (Hartshorne and Pyun, 1971; Eisenberg and Kielley, 1970). Excess pure actin fully activates the ATPase of an actomyosin which has only an actin-linked regulatory system by preferentially binding to myosin, even in the absence of calcium ions. The ATPase activity of a muscle which has a myosin-linked regulatory system is not stimulated by actin in the absence of calcium, since in this system myosin cannot combine with pure actin. Troponin or tropomyosin is not required for myosin control. The MgATPase activity of lobster actomyosin is activated by excess pure actin in the absence of calcium to the same level as in the presence of calcium, indicating not only the presence of an actin-linked regulation, but also the absence of a functional regulation which acts on myosin (Table I). Furthermore, if lobster regulatory proteins are added to rabbit actomyosin, the MgATPase is inhibited in the absence of calcium, and this inhibition is reversed by pure rabbit actin. Excess rabbit myosin does not affect the system.

The absence of a myosin-linked regulatory system is also suggested by the fact that purified lobster myosin does not form a calcium sensitive complex when combined with pure rabbit actin, and it does not bind calcium at concentrations where calcium regulates.

The calcium sensitivity of lobster myofibrils and actomyosin preparations is readily lost by dialyzing these preparations extensively against water (Hartshorne and Perry, 1967). Concurrent with the loss of calcium sensitivity, protein components are solubilized from the myofibrils and actomyosin. This supernatant contains several different polypeptides as shown by SDS acrylamide gel electrophoresis, including tropomyosin and presumably the other regulatory

[2] % calcium sensitivity = $[1 - (Mg\text{-}ATPase_{EGTA}/Mg\text{-}ATPase_{Ca})]100$.

Table I: Competitive Actin Binding.

Proteins	Mg-ATPase Ca^{2+} (μmol of ATP per min per mg)[a]	% Calcium Sensitivity[b]
0.2 mg of lobster myofibril	0.40	76
0.2 mg of lobster myofibrils + 0.4 mg of rabbit actin	0.44	6
0.18 mg of lobster natural actomyosin	0.39	44
0.18 mg of lobster natural actomyosin + 0.4 mg of rabbit actin	0.40	~0
0.15 mg of lobster regulatory proteins + rabbit actomyosin (0.96 mg of myosin to 0.24 mg of actin)	0.23	74
+ 0.3 mg of rabbit actin	0.23	~0
+ 0.8 mg of rabbit myosin	0.23	65

[a] Conditions of ATPase measurements: 0.03 M NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM ATP without and then with the addition of 0.2 mM $CaCl_2$ in 10 ml final volume. Specific activity is calculated for the initial amounts of myofibril or actomyosin. [b] $[1 - (\text{Mg-ATPase}_{EGTA}/\text{Mg-ATPase}_{Ca^{2+}})]100 = .$

Table II: Stoichiometry of Regulatory Proteins and Actin.

Component	Moles of Actin/Mole of Component[a] Range[b]	Mean[b]
TN-C	7.4–11.7	10.3
TN-I	3.4–5.5	4.6
TN-T	5.7–10.9	8.1
Tropomyosin	3.8–7.1	5.3

[a] Ratio of the components of the regulatory proteins to rabbit actin at a point where the regulatory proteins cause maximum inhibition of the actomyosin ATPase activity (*cf.* arrow on Figure 3). Concentration of actin and regulatory proteins was obtained from protein determinations; concentration of the regulatory protein components was obtained from the densitometry of 12% sodium dodecyl sulfate acrylamide gels stained by Fast Green, assuming that the proteins give an equal color yield. The following molecular weights were used: actin, 43,000; TN-C, 17,000; TN-I, 32,000; TN-T, 52,000; tropomyosin, 68,000. (The two individual polypeptides of tropomyosin move anomalously slow on gels, corresponding to an apparent chain weight of about 38,000.) [b] Data from six experiments. Rabbit regulatory proteins all give a mean value of approximately 7 (Potter, 1974).

proteins. When pure rabbit actin is added to the supernatant and centrifuged at high speed, tropomyosin and these other components will cosediment with actin. If the supernatant is added to rabbit synthetic actomyosin, it confers calcium sensitivity on the actomyosin MgATPase, indicating that the system now contains the full complement of regulatory proteins. Only three major components, in addition to actin and tropomyosin, are detected by acrylamide gel electrophoresis in the fraction of the supernatant which precipitates between 0.35 and 0.7 ammonium sulfate saturation (Figure 2a). These three major components along with tropomyosin will be shown to be responsible for control function and to be analogous to vertebrate troponin. The behavior of lobster actomyosin and myofibrils during the above preparation and the pCa profiles of their MgATPase activity are analogous to rabbit preparations, and suggest that the main features of regulation in the two organisms may be similar.

Components of Lobster Relaxing Proteins. The crude preparation of regulatory proteins may contain up to six major components with an approximate apparent chain weight of about 60,000 (variable), 52,000, 38,000, 27,000, 17,000, and a clearly discernible doublet of 30,000–32,000 was examined by sodium dodecyl sulfate acrylamide gel electrophoresis. (Figure 1a shows all the components that are found on natural actomyosin.) The 38,000 component was identified as tropomyosin. In our hands both lobster and rabbit tropomyosin have anomalously slow mobilities and the molecular weights obtained from gel electrophoresis are too high. Lobster tropomyosin, unlike rabbit tropomyosin, moves as a single band. Some of the other components, in particular, the 17,000, the 32,000 doublet, and the 52,000 components appear to be the subunits of troponin. (In addition, a 27,000 chain weight component may also be seen; it is not found in myofibrils and is likely a breakdown product (Figure 1a and b).)

The molar ratios of troponin components were approximated from the densitometry of SDS acrylamide gels, assuming equal color yield (Gorovsky *et al.*, 1970). At the smallest ratio of troponin to actin giving maximum calcium sensitivity (about 0.12 mg of regulatory protein per 1 mg of actomyosin (A/M 1:4) (Figure 3)), one estimates that there is 1 mol of 32,000 doublet, 0.7–1.0 mol of 52,000, and about 0.5 mol of 17,000 component for a mol of tropomyosin. The ratio of actin to tropomyosin is 4–6 (Table II).

Tropomyosin. This component can be obtained in a relatively pure form by the procedures developed for preparation of rabbit tropomyosin. It forms aperiodic paracrystals when precipitated in magnesium at pH 8.0 or periodic paracrystals at pH 5.2 with ammonium sulfate present. The periodic structures of the paracrystals obtained by acid precipitation resemble those obtained with rabbit tropomyosin precipitated at pH 8.0 with magnesium (Cohen and Longley, 1966) (Figure 4). The repeat period, however, may be about 3% shorter than that characterizing rabbit tropomyosin. Furthermore, on sodium dodecyl sulfate acrylamide gel electrophoresis, lobster tropomyosin moves slightly faster than rabbit tropomyosin, again suggesting that it may have a slightly lower molecular weight. The amino acid compositions of the lobster and rabbit tropomyosins are rather similar; neither tropomyosin contains proline residues (Table V).

Tropomyosin is a required component for the regulation of lobster muscle. In its absence, lobster troponin has no effect on the ATPase activity of rabbit actomyosin (Table III). Rabbit tropomyosin can replace lobster tropomyosin and form a functional complex with lobster troponin. It has been shown previously that clam tropomyosin from *M. mercenaria* interacts with rabbit troponin (Lehman *et al.*, 1972), and that rabbit tropomyosin interacts with insect

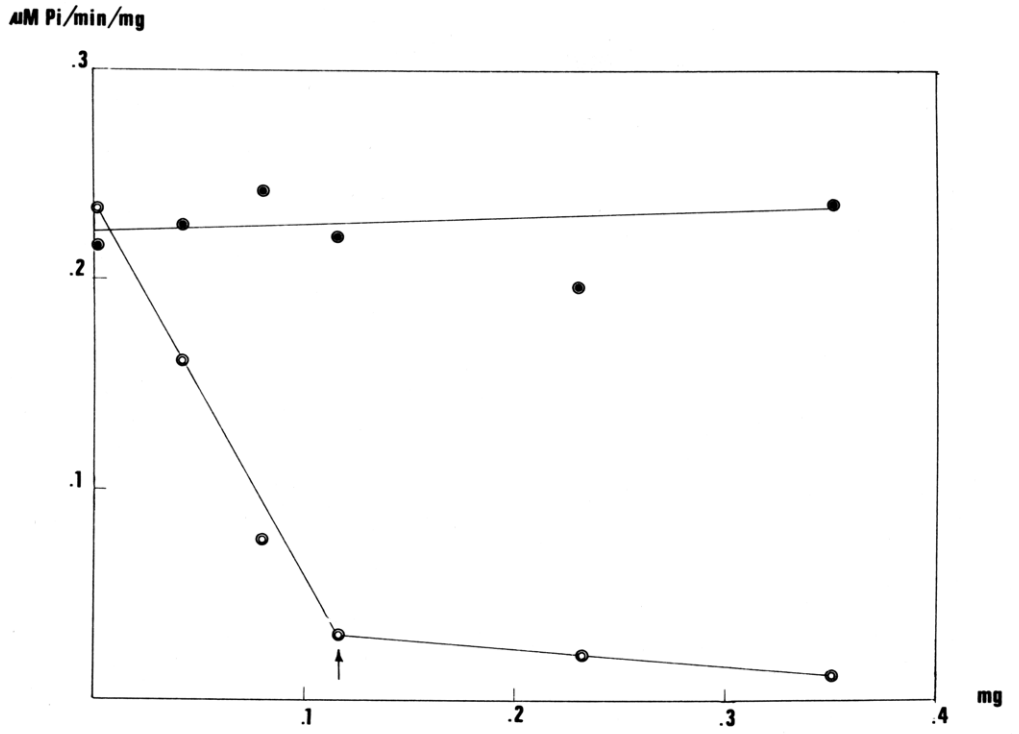

FIGURE 3: The effect of regulatory proteins on the ATPase activity of rabbit actomyosin. Increasing amounts of lobster regulatory protein are added to an actomyosin prepared by mixing 0.8 mg of rabbit myosin with 0.2 mg of rabbit actin, and the ATPase is measured in the absence (◎) and then in the presence of calcium (◉). 0.03 M NaCl, 1 mM $MgCl_2$, and 0.1 mM EGTA without and with 0.2 mM $CaCl_2$. Arrow indicates amount of regulatory protein needed for full inhibition.

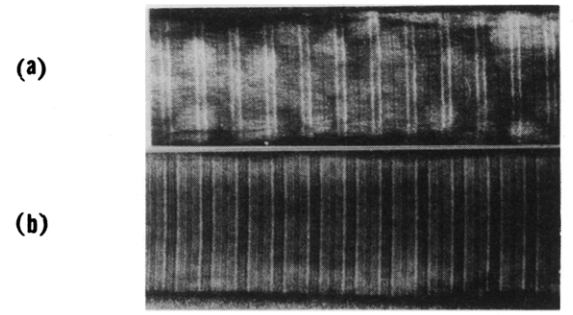

FIGURE 4: Tropomyosin paracrystals. (a) Lobster tropomyosin precipitated in 0.01 M sodium acetate (pH 5.2) and 0.12 M ammonium sulfate. (b) Rabbit tropomyosin precipitated in 0.05 M Tris (pH 8.0) and 0.02 M $MgCl_2$. Negatively stained with 1% uranyl acetate. Photographs taken at the same magnification without altering the setting of the microscope. Note that the periodicity of lobster tropomyosin is slightly less.

Table III: Effect of the Components of the Lobster Regulatory Proteins on the ATPase Activity of Rabbit Actomyosin.

Expt	Additions to Actomyosin[a]	w/w Ratios	Mg-ATPase$_{EGTA}$[b]	Mg-ATPase$_{Ca}$[b]
A	Troponin	1:0.28	1.07	0.88
	Troponin + tropomyosin	1:0.28:1.3	0.01	0.97
B	None		0.48	0.43
	Tropomyosin	1:0.2	0.49	0.45
	Tropomyosin + TN-I	1:0.2:0.14	0.12	0.14
	Tropomyosin + TN-I + TN-C + TN-T	1:0.2:0.05: 0.056: 0.014	0.05	0.29

[a] 0.24 mg of actomyosin: 0.18 mg of myosin and 0.06 mg of actin. [b] μmol of ATP hydrolyzed per min per mg of actomyosin at 22° in 0.03 M NaCl, 1 mM $MgCl_2$, 0.5 mM ATP, and 0.1 mM EGTA without and then with 0.2 mM $CaCl_2$.

troponin from *Lethocerus maximus* (Bullard *et al.*, 1973) and *Limulus* troponin (Lehman *et al.*, 1972).

A 60,000 component is occasionally found in the crude preparation of regulatory proteins. This component coprecipitates with tropomyosin during the initial acid precipitation but it is lost during the subsequent purification of tropomyosin and it does not appear to be a necessary component of regulation.

Troponin. The regulatory proteins are separated into two fractions with the aid of a SE-Sephadex column. The 52,000 and 17,000 components are eluted at low ionic strength in 6 M urea. These two components can be further fractionated by the selective precipitation of the 52,000 component at low ionic strength in the presence of ATP (Figures 1d and e, and 2d and e). The 32,000 doublet component elutes from the SE-Sephadex at higher ionic strength together with a 27,000 component (Figures 1c and 2b). The 27,000 component becomes insoluble after trichloroacetic acid precipitation or boiling for 10 min (Figure 2b and c). We have not been able to separate the two components of the doublet 32,000 band.

The 32,000 component of lobster troponin has properties similar to rabbit TN-I. It inhibits the ATPase activity of rabbit actomyosin both in the presence and in the absence of calcium (Table III). Tropomyosin appears to be necessary for this inhibition. In its absence TN-I from lobster has little effect on the ATPase activity even when added in excess (0.6 mg of TN-I/mg of actomyosin). The inhibitory effect remains after 10 min of boiling or precipitation with

Table IV: Calcium Binding by the Components of the Regulatory Proteins.

Protein	Binding (nmol of Ca bound/mg of Protein)[a] Range (nmol)	Mean (nmol)	Number of Measurements
Regulatory proteins	1.23–4.61	2.88	12
Tropomyosin	0.18–0.98	0.54	5
Troponin	4.48–7.43	6.54	5
TN-I	0	0	2
TN-C	5.77–6.95	6.36	2

[a] By equilibrium dialysis in 0.04 M NaCl, 10 mM imidazole-HCl (pH 7.0), 1 mM $MgCl_2$, 0.02–0.04 mM EGTA, and CaEGTA to give free calcium concentrations of 5×10^{-7} to 1×10^{-5} M.

7.5% trichloroacetic acid, although the 27,000 component is lost, indicating that the latter is an impurity (Figure 2c). Since the 27,000 component is not present in the initial actomyosin preparation (Figure 1a), it is likely to be a breakdown product. One notes that the 32,000 doublet can also be observed in *Limulus* relaxing proteins and on thin filaments of a number of invertebrate muscles (Lehman *et al.*, 1972). It will cosediment with actin even in the absence of tropomyosin.

The 17,000 component has properties similar to TN-C of rabbit. It binds calcium, and in the presence of calcium it reverses the inhibitory action of TN-I (Tables III and IV). In the absence of TN-I, TN-C has no effect on the ATPase activity of actomyosin. Although lobster TN-C seemingly functions as rabbit TN-C and is the only component which binds calcium with high affinity in the presence of magnesium, the calcium binding of lobster TN-C is significantly less than that of rabbit TN-C. The binding by isolated lobster TN-C amounts of 0.2–0.4 mol of calcium/17,000 g which contrasts to rabbit preparations which may bind up to 4 mol of calcium/17,000 g (Hartshorne and Pyun, 1971; Greaser *et al.*, 1972; Weber and Murray, 1973). It is unlikely that the lesser calcium binding is due solely to loss of binding sites during preparation since the preparations are functional in an ATPase assay. Unfractionated preparations of regulatory proteins bound between 1.2 and 4.6 nmol of calcium/mg of protein. Later preparations showed the higher binding. Taking the largest binding and assuming a mole to mole ratio of tropomyosin-TN-C-Tn-I-TN-T (Potter, 1974), the binding would amount to 0.9 mol of calcium by a mol of TN-C. It is noteworthy that these preparations fully regulate the ATPase activity of actomyosin at a stoichiometry of 6 mol of actin to 1 mol of troponin-tropomyosin (Figure 2), and therefore it is unlikely that they contain significant amounts of inactive proteins. The exact stoichiometry of the lobster troponin subunits remains to be established. One also notes that washed lobster myofibrils bind significantly smaller amounts of calcium than those of rabbit, 1–1.5 μmol of calcium/g of lobster myofibril and 3–4 μmol of calcium/g of rabbit myofibrils (*cf.* Weber and Murray, 1973; Kendrick-Jones *et al.*, 1970). It appears thus that concentration in lobster muscles may be triggered by fewer calcium ions bound on troponin.

We have some evidence that the 52,000 component corresponds to TN-T and can combine with tropomyosin. The interpretation of the experiments on the interactions of this protein are made difficult by its limited solubility. TN-C solubilizes TN-T and its presence is necessary for these experiments. TN-T appears to form a complex with tropomyosin which sediments slower than tropomyosin alone in 0.6 M NaCl. The effect, however, is considerably dependent on ionic strength and disappears in the case of lobster at 1 M NaCl. TN-T (with TN-C present) coprecipitates with tropomyosin at 8 mM $MgCl_2$ and 0.05 M Tris (pH 8.0). Pure lobster tropomyosin requires about 0.02 M $MgCl_2$ for precipitation. Periodic structures are not seen on either the tropomyosin-TN-T paracrystals or the tropomyosin paracrys-

Table V: Percentage Composition of Regulatory Protein Components for Fourteen Amino Acids.

Amino Acid	Troponin T		Troponin I		Troponin C		Tropomyosin		
	Rabbit[a]	Lobster	Rabbit[a]	Lobster[b]	Rabbit[c]	Lobster[d]	Rabbit[e]	Lobster[f]	Lobster[g]
Lys	13.0	15.0	13.2	16.0	6.8	9.4	13.4	11.6	9.8
His	2.1	1.0	2.1	0.7	1.2	1.2	0.7	0.5	0.4
Arg	9.0	8.1	8.3	7.9	5.1	4.2	5.1	7.7	7.9
Asp	8.7	8.9	10.2	10.7	13.4	11.3	11.1	13.0	12.6
Thr	2.3	2.8	2.2	2.9	3.5	3.8	3.5	2.5	3.4
Ser	3.6	4.1	5.7	2.9	4.5	3.2	5.0	2.5	5.4
Glu	25.0	28.4	20.5	20.5	23.4	18.1	26.5	27.9	25.0
Pro	3.6	4.2	3.0	3.2	2.4	4.9	0.2	0.0	3.8
Gly	3.4	6.0	5.3	6.0	7.5	15.5	1.5	2.2	1.8
Ala	11.0	5.6	9.3	9.5	9.5	7.7	13.5	11.5	11.3
Val	4.5	2.7	4.7	3.7	5.3	5.9	3.4	4.7	4.1
Ile	3.1	3.0	2.8	4.9	5.4	4.3	3.8	2.5	1.5
Leu	7.8	8.1	10.9	8.1	6.8	7.4	11.9	12.1	11.0
Phe	1.8	2.1	1.0	2.8	5.1	3.4	0.4	1.4	1.4

[a] Wilkinson *et al.*, 1972. [b] Average of three runs, three different preparations. [c] Schaub *et al.*, 1972 and Hartshorne and Pyun, 1971. [d] Average of two runs. [e] Kominz *et al.*, 1957a. [f] Average of two runs. [g] Kominz *et al.*, 1957b.

tals. Tropomyosin is required to cosediment TN-T with actin.

The amino acid compositions of the individual components show a similar general pattern between the troponin subunits of lobster and rabbit. Differences, however, are also seen. Thus, the proline and glycine content of lobster TN-C appears to be considerably greater compared to rabbit, while the phenylalanine and isoleucine residues appear to be fewer. Smaller variations are found among a number of other residues (Table V).

Discussion

The composition and properties of lobster troponin indicate an essential similarity between vertebrate and invertebrate troponins. Lobster troponin consists of two, possibly three, different subunits, including an inhibitory and a calcium binding component which evidently correspond in function and role to TN-C and TN-I of vertebrate troponins. The third component of lobster troponin is less well characterized. There is evidence, however, that it can interact with tropomyosin and it is likely that it corresponds to TN-T in rabbit. One should realize, however, that proteins, other than the regulatory proteins, may interact with the thin filaments (*cf.* Tilney *et al.*, 1973), and further evidence is needed to establish that the 52,000 protein is TN-T. The requirement for tropomyosin in actin-linked calcium regulation is a further indication that vertebrate and invertebrate troponins may function in a similar fashion (Lehman *et al.*, 1972; Bullard *et al.*, 1973). It appears that, for an actin-linked regulation, the presence of several subunits is required. These properties then may be a generalized feature of systems which regulate contraction along the thin filaments.

Lobster tropomyosin and TN-C have a lower molecular weight than their vertebrate counterparts. In contrast, TN-I and particularly TN-T have greater molecular weights than those found in rabbit and chicken. Although there is a similarity in the amino acid composition of lobster and rabbit troponin subunits, the subunits are clearly not identical. Despite these differences, there are some indications of an interaction between lobster and rabbit regulatory proteins. Lobster troponin–tropomyosin can react with rabbit actin and lobster troponin with rabbit tropomyosin and actin. Although hybrids can be formed between tropomyosin and troponin of vertebrate and invertebrate muscles, hybridization of troponin subunits has not been tested yet.

It is noteworthy that the lobster's regulatory response requires fewer calcium ions than rabbit's (Potter *et al.*, 1974). There is at most 1 mol of calcium bound/mol of TN-C. Calcium binding by myofibrils is also low and agrees with the notion that cooperativity of several calcium binding sites within an actin thin filament unit in lobster regulation is unlikely (*cf.* Bremel and Weber, 1972).

Throughout, we have also assumed that the doublet bands at a position corresponding to 32,000 on SDS acrylamide gels are isomorphic forms of TN-I and that both may represent the inhibitory component of troponin. Visual observation indicates that the two bands are in approximately equal concentrations, the lower band perhaps generally slightly higher. We note that Limulus troponin also has a similar doublet with a chain weight of about 32,000 with the upper band more intense (W. Lehman, personal communication). Moreover, a number of thin filament preparations from arthropods have such doublet bands (Lehman *et al.*, 1972). Of the three troponin components, TN-T appears to be the most variable one in chain weight, ranging from 37,000 to 60,000 in animals examined to date (Lehman *et al.*, 1972).

Thus, the multicomponent nature of regulation is similar in vertebrates and crustaceans. So far, the low calcium binding (moles of Ca/mole of TN-C) appears to be characteristic of all invertebrate troponins but is not found with any of the vertebrates (*cf.* Potter *et al.*, 1974). The overall results support a view that actin-linked regulation requires interactions between different subunits and is dependent on the presence of tropomyosin.

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The *Bombyx mori* Silk Proteins: Characterization of Large Polypeptides†

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ABSTRACT: Proteins taken directly from the *Bombyx mori* silk gland have been separated and identified as either fibroin or sericin on the basis of their location within the gland and their amino acid composition. Molecular weights of these polypeptides have been determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and by agarose-guanidine chromatography. Fibroin consists of approximately equimolar amounts of two large (350,000) polypeptide chains. These may be the products of distinct fibroin alleles present in hybrid silkworm strains. Sericin, on the other hand, is composed of at least the three largest polypeptides (130,000–220,000) present in a mixture of proteins ranging in size from about 20,000 to 220,000.

Physical characterization of the silk proteins produced by *Bombyx mori,* the commercial silkworm, is of current interest because recent experiments have revealed some structural features of the gene and the mRNA molecule coding for one of these proteins, fibroin (Suzuki and Brown, 1972; Suzuki *et al.,* 1972; Lizardi and Brown, 1973).

Historically, two kinds of silk proteins have been distinguished: fibroin, a fibrous protein which makes up the core of the silk filament; and sericin, a poorly characterized protein (or proteins) which surrounds the spun fiber and functions as an adhesive (Lucas *et al.,* 1958). Both types of protein accumulate in the middle silk gland during the fifth larval instar. Classical experiments in which specific portions of the gland were removed from living animals establish that fibroin is actually synthesized by the posterior silk gland, whereas sericin is produced by the cells of the middle silk gland (Machida, 1927).

Although fibroin from *Bombyx mori* has been studied for over a century (Cramer, 1865), accurate determination of its size and possible subunit structure has been complicated by the difficulty of preparing solutions in which the molecules are disaggregated yet intact. Hence, molecular weight estimates for fibroin, which have been obtained primarily by sedimentation analysis, range from about 2×10^4 to over 10^6 (Rao and Pandit, 1965; Lucas, 1966; Tashiro and Otsuki, 1970a,b; Tashiro *et al.,* 1972; Tokutake and Okuyama, 1972; Sridhara *et al.,* 1973; Sasaki and Noda, 1973a,b). It is not clear whether more than one type of polypeptide chain is involved. The inability to distinguish clearly between fibroin and sericin components has contributed further uncertainty to the characterization of these molecules.

In this paper, I present evidence that fibroin consists of approximately equimolar amounts of two large (3.5×10^5) polypeptide chains which are distinct from the sericin proteins. Rather than attempting solubilization of spun silk fibers (a procedure which frequently leads to degradation), I have solubilized fibroin and sericin taken directly from the middle silk gland of mature *Bombyx* larvae. Fractionation and characterization of the silk proteins were achieved by molecular sieve techniques (SDS[1] polyacrylamide gel electrophoresis and guanidine-agarose chromatography).

Materials and Methods

Rearing of Silkworms. Silkworm larvae were hatched from a laboratory stock of eggs (originally supplied by O. Yamashita) and were reared either on fresh mulberry leaves or on the artificial diet described by Suzuki and Brown (1972). In both cases, the larvae weighed between 4

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; Gdn · HCl, guanidine hydrochloride.