

Structural Changes Accompanying Phosphorylation of Tarantula Muscle Myosin Filaments

Roger Craig,* Raúl Padrón,† and John Kendrick-Jones‡

*Department of Anatomy, University of Massachusetts Medical School, Worcester, Massachusetts 01655;

†Laboratorio de Biofísica del Músculo, Instituto Venezolano de Investigaciones Científicas (IVIC), Apdo 21827, Caracas 1020A, Venezuela; and ‡Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom

Abstract. Electron microscopy has been used to study the structural changes that occur in the myosin filaments of tarantula striated muscle when they are phosphorylated. Myosin filaments in muscle homogenates maintained in relaxing conditions (ATP, EGTA) are found to have nonphosphorylated regulatory light chains as shown by urea/glycerol gel electrophoresis and [³²P]phosphate autoradiography. Negative staining reveals an ordered, helical arrangement of crossbridges in these filaments, in which the heads from axially neighboring myosin molecules appear to interact with each other. When the free Ca²⁺ concentration in a homogenate is raised to 10⁻⁴ M, or when a Ca²⁺-insensitive myosin light chain kinase is added at low Ca²⁺ (10⁻⁸ M), the regulatory light chains of myosin become

rapidly phosphorylated. Phosphorylation is accompanied by potentiation of the actin activation of the myosin Mg-ATPase activity and by loss of order of the helical crossbridge arrangement characteristic of the relaxed filament.

We suggest that in the relaxed state, when the regulatory light chains are not phosphorylated, the myosin heads are held down on the filament backbone by head-head interactions or by interactions of the heads with the filament backbone. Phosphorylation of the light chains may alter these interactions so that the crossbridges become more loosely associated with the filament backbone giving rise to the observed changes and facilitating crossbridge interaction with actin.

CONTRACTION in motile systems is controlled by Ca²⁺-dependent regulatory protein switches associated with the myosin or actin filaments. At low Ca²⁺ levels, actin-myosin interaction is inhibited and actin-activated myosin ATPase activity is low, whereas at high Ca²⁺ levels, inhibition of actin-myosin interaction is removed and the myosin ATPase is activated by actin. In the case of myosin-linked regulation, which occurs in many muscle and non-muscle cells, Ca²⁺ regulation is mediated by small polypeptide chains (regulatory light chains) that are present on the myosin heads (Kendrick-Jones et al., 1976; Kendrick-Jones and Scholey, 1981). Two mechanisms of myosin-linked regulation have been described. In one, exemplified by scallop striated muscle, activation of the myosin MgATPase by actin requires binding of Ca²⁺ to the myosin heads (Kendrick-Jones et al., 1970; Szent-Györgyi et al., 1973). In the other, the myosin filaments are switched "on" (enabled to interact with actin) by Ca²⁺-induced phosphorylation of the regulatory light chains. This mechanism occurs in vertebrate smooth muscle and nonmuscle cells (Adelstein and Eisenberg, 1980; Kendrick-Jones and Scholey, 1981; Small and Sobieszek, 1980; Watanabe, 1985) and has also been reported to operate in the striated muscle of the horseshoe crab, *Limulus* (Sellers, 1981).

The structural changes that occur in myosin filaments

when they are switched "on" (but before their interaction with actin) are poorly understood. The problem is difficult to study by X-ray diffraction of contracting muscle since when thick and thin filaments overlap, the effects of interaction of the crossbridges with actin will be superimposed on the changes in the thick filaments themselves. However, preservation of the three-dimensional crossbridge array in isolated negatively stained relaxed thick filaments (e.g., Kensler and Levine, 1982; Vibert and Craig, 1983; Crowther et al., 1985) provides a starting point for observing such changes by electron microscopy. Our most detailed knowledge of thick filament structure in the "off" (relaxed) state has come from three-dimensional reconstruction of negatively stained filaments from the leg muscles of the tarantula. Relaxed tarantula thick filaments have a helically ordered arrangement of crossbridges in which the heads of individual myosin molecules appear to point in opposite directions along the filament such that interactions occur between the heads of axially adjacent myosin molecules (Padrón et al., 1984; Crowther et al., 1985). On the basis of this structure we suggested (Crowther et al., 1985) that head-head interaction or interaction of the heads with the backbone locked the crossbridges close to the thick filament backbone, helping to inhibit interaction with actin and to maintain the "off" state. Here we test the hypothesis that switching "on" of tarantula thick filaments

(a) depends on phosphorylation of the myosin regulatory light chains (by analogy with the closely related *Limulus* muscle [Sellers, 1981]), and (b) involves a weakening of the head-head or head-backbone interactions, which could thus facilitate interaction of crossbridges with actin (cf., Vibert and Craig, 1985).

These results have been reported in preliminary form (Craig et al., 1985).

Materials and Methods

Red-legged (*Brachypelma*) and pink-foot (*Avicularia*) tarantulas were obtained from local pet shops. ATP and ATP- γ -S were obtained from Boehringer Corp., Ltd., Lewes, Sussex; trypsin (from bovine pancreas) and trypsin inhibitor (soya bean) from Sigma Chemical Co., Ltd., Poole, Dorset; and Coomassie PAGE Blue 83 was from British Drug Houses, Ltd., Poole, Dorset.

Biochemical Procedures

Myosin light chain kinase (MLCK)¹ was prepared from chicken gizzards by the procedure of Adelstein and Klee (1981) as described previously (Kendrick-Jones et al., 1983). Unregulated kinase was prepared by tryptic digestion of MLCK in the presence of bound calmodulin (Adelstein et al., 1981). Kinase (0.5 mg/ml) in 100 mM NaCl, 25 mM Tris-HCl, pH 7.5, 0.2 mM CaCl₂, 2 mM dithiothreitol (DTT) together with excess calmodulin was digested with trypsin (at a 250:1 protein to enzyme wt/wt ratio) for 1 h on ice. Digestion was terminated with a 10-fold excess of trypsin inhibitor. Calmodulin was prepared from bovine brain as described previously (Scholey et al., 1981). Tropomyosin and actin were prepared from rabbit skeletal muscle by the methods of Bailey (1948) for tropomyosin and Feuer et al. (1948) for actin, with the minor modifications described by Kendrick-Jones et al. (1970) to remove troponin and tropomyosin contamination. Overloaded SDS-polyacrylamide gels of each actin preparation gave a single band at 42 kD (>98% purity). Myosin was prepared from glycerinated tarantula muscle by the procedure (modified from that devised by Focant and Huriaux, 1976), previously used to prepare gizzard myosin (Kendrick-Jones et al., 1983), but with the following additional modifications. The muscle was rinsed to remove glycerol and was finely minced with scissors. It was homogenized with a Polytron (Kinematica, Luzern, Switzerland) for four 5-s bursts in 50 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 10 mM phosphate buffer, pH 7.0, 0.5 mM DTT, 2 mM Na₂S₂O₈, 75 mg/liter phenylmethylsulfonyl fluoride (PMSF), 20 mg/liter L-1-tosylamide-2-phenyl-ethyl-chloromethylketone (TPCK), 20 mg/liter benzyl arginylethyl ester, 10 mg/liter trypsin inhibitor, 1 mg/liter leupeptin, and was centrifuged at 7,000 rpm for 10 min. The "washing" procedure was repeated twice more and the washed muscle (myofibrils) extracted with high salt solution (0.6 M NaCl, 1 mM EGTA, 10 mM ATP, 2 mM MgCl₂, 20 mM phosphate buffer, pH 7.0, 10 mM Tris-HCl buffer, pH 7.5, 1 mM DTT) by stirring for 10 min on ice. The actomyosin supernatant was obtained by centrifugation at 20,000 rpm (56,000 g) for 20 min. MgCl₂ was added to a final concentration of 22 mM at pH 7.5, and the myosin was purified from this actomyosin by ammonium sulphate fractionation (between 37 and 65% saturation) as described previously for gizzard myosin (Kendrick-Jones et al., 1983). The myosin was reprecipitated by dialysis against 20 mM NaCl, 25 mM imidazole, pH 6.5, 5 mM MgCl₂, 0.25 mM DTT, 0.1 mM PMSF, redissolved in 1 M NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM DTT (at ~10 mg/ml), and clarified at 100,000 g for 1 h before use. It was stored in ice and used within 3 d. One myosin preparation was further subjected to gel filtration on Sepharose CL4B under the conditions described in Scholey et al. (1982) to remove the last trace of Ca²⁺-sensitive kinase activity. No significant differences in the PAGE patterns or ATPase activities of the myosin preparation before and after chromatography were detected.

Protein concentrations were estimated spectrophotometrically using absorption coefficients ($A_{280}^{1\text{mg/ml}}$) of 0.56 for myosin and 1.38 for MLCK (Adelstein and Klee, 1981), 1.08 for actin and 0.38 for tropomyosin (Greaser and Gergely, 1971). "Myofibril" or "filament suspension" concentrations were measured after dissolving them in 1% SDS, pH 7.5, and boiling for 1 min, using an $A_{280}^{1\text{mg/ml}}$ of 0.7.

For the ATPase assays, the myosin (5 mg/ml) in 150 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 0.5 mM DTT, 10 mM Tris-HCl, pH 7.5, was phos-

phorylated (or thiophosphorylated) using 30 μ g/ml calmodulin, 25 μ g/ml MLCK, and 2.5 mM MgATP (or 1 mM MgATP- γ -S) at 25°C for 15 min.

Since the filament suspensions used in the electron microscopic studies contained high levels of endogenous calmodulin and MLCK, phosphorylation was carried out in these experiments by the addition of Ca²⁺ (0.1 mM final concentration of free Ca²⁺) to the filaments in relaxing medium followed by incubation at 25°C for 5–30 min. Alternatively, these filament suspensions were phosphorylated in relaxing medium (absence of Ca²⁺) by the addition of trypsin-treated Ca²⁺-insensitive MLCK (50 μ g/ml) followed by incubation at 25°C for 5–30 min.

The level of phosphorylation of the "regulatory" light chains in the filament preparations assayed under various conditions (see Fig. 2) was monitored by the change in their mobility on 40% glycerol/urea PAGE. Aliquots of the filament suspensions were taken at fixed time points into 1.5-ml Eppendorf tubes and treated with ice cold TCA (3% final concentration). The precipitated protein was centrifuged, washed with 1 ml of ice cold acetone, and rapidly "dissolved" in a freshly prepared solution of 9 M urea, 50 mM Tris-300 mM glycine, 5 mM DTT, 0.01% Bromophenol blue (final pH = 8.6) with agitation or brief sonication. The light chains are readily soluble in the urea solution whereas the myosin heavy chains, paramyosin, actin, etc., are only partially soluble. The samples were immediately run on 10% acrylamide-40% glycerol, 20 mM Tris-122 mM glycine, pH 8.6, gels (Perrie and Perry, 1970). As a further control, the purified myosin samples were also subjected to the TCA/acetone wash procedure.

Microslab SDS gradient PAGE was performed by the method of Matsudaira and Burgess (1978). Aliquots from the filament suspensions were taken at fixed time points, mixed 1:1 with SDS sample buffer, boiled, and at the end of the assay (i.e., within approximately 30 min) run on gels, together with the control samples and the following molecular mass standards: 200 kD, myosin heavy chains; 94 kD, phosphorylase; 68 kD, BSA; 42 kD, actin; 25 kD, rabbit skeletal muscle myosin LC, light chain; 19 kD, rabbit myosin "regulatory" LC₂ light chain.

Both types of gels were stained with 0.5% Coomassie Blue (PAGE Blue 83) in 50% methanol, 10% acetic acid (the glycerol/urea gels take longer to stain/densify and are less sensitive than the SDS gels). They were rapidly destained and dried, and if [³²P]- γ -ATP (5 μ Ci/ μ mol) had also been used (to monitor phosphorylation and demonstrate that the change in mobility was due to light chain phosphorylation), then the gels were autoradiographed in cassettes fitted with calcium/tungstate/phosphor screens (Cronex Lightning Plus; Dupont Co., Wilmington, DE) at -70°C using pre-fogged film to increase sensitivity. We are thus able to detect faint traces of kinase activity in our myosin preparations.

The actin-activated MgATPase activities of the myosin preparations were routinely measured at 25°C by the pH-stat assay procedure (Kendrick-Jones et al., 1976), but were also assayed using the isotopic [³²P]phosphate procedure (Scholey et al., 1981).

Electron Microscopy

The compositions of the solutions used to prepare the filament suspensions for electron microscopy and for measuring their level of light chain phosphorylation were as follows.

Relaxing Medium. 0.1 M NaCl, 8 mM MgCl₂, 5 mM ATP, 1 mM EGTA, 3 mM Na₂S₂O₈, 10 mM NaP_i, pH 7.0.

Activating Medium. Relaxing medium + 1.1 mM CaCl₂, pH 7.0.

Relaxing Rinse. 0.1 M Na acetate (Ac), 2 mM MgAc, 1 mM ATP, 0.2 mM EGTA, 3 mM Na₂S₂O₈, 2 mM imidazole, pH 7.0.

Activating Rinse. Relaxing rinse + 0.3 mM CaCl₂, pH 7.0.

Filament homogenates were prepared from chemically skinned tarantula muscles as described by Crowther et al. (1985), with the following modifications. (a) The concentration of EGTA in the relaxing medium was 1 (not 5) mM. (b) The precaution of using a cocktail of proteolytic enzyme inhibitors was found to be unnecessary for good structural preservation and was omitted. (c) Muscles were homogenized in a Polytron homogenizer. Muscles, stored in relaxing medium on ice, were used within 3 d of removal from the animal, and once homogenized, experiments were completed on them within about 2 h to minimize structural deterioration and uncontrolled phosphorylation, which occurred with longer storage.

Negative staining with 6–10 drops of 1% (wt/vol) uranyl acetate was carried out at room temperature, as described by Crowther et al. (1985), on filament suspensions in which the state of phosphorylation of the regulatory light chains was monitored as described above. Aliquots of the filament suspension were removed for electron microscopy at the same time points as those for the phosphorylation assays and were diluted 20-fold into, and kept in, activating medium (or relaxing medium for "relaxed" controls) at 0°C.

1. Abbreviation used in this paper: MLCK, myosin light chain kinase.

Since phosphorylation has a high Q_{10} , this should have essentially halted further phosphorylation until the grids were made, generally within 30 min. Immediately before staining, the grid was washed with ~ 10 drops of relaxing rinse or activating rinse. All micrographs are of filaments suspended in unbroken films of stain over holes in the carbon substrate (Vibert and Craig, 1982, 1983). Micrographs were recorded with normal electron doses, at a nominal magnification of $26,000\times$, on a JEOL 100CX electron microscope operated at 80 kV with an anticontamination cold finger.

Results

Thick Filaments in Relaxing Medium

Muscle homogenates, containing suspensions of thick and thin filaments, were incubated in relaxing medium at 25°C and after 5, 10, and 30 min, drops were removed for electron microscopy and aliquots taken to determine the level of light chain phosphorylation. To avoid the deleterious effects of staining thick filaments in the presence of Cl^- ions (Crowther et al. 1985), grids were rinsed for 5–10 s with 6–10 drops of relaxing rinse (in which the major anion is acetate) immediately before staining. The majority of thick filaments observed showed a clear helical organization of crossbridges (Fig. 1) as reported previously (Levine et al., 1983; Crowther et al., 1985). The diameter of the filaments to the outer edges of the crossbridges was ~ 36 nm. The appearance of the helical structure was independent of the length of time (up to 30 min) of incubation in relaxing medium, and gel electrophoresis demonstrated that the light chains remained as shown in Fig. 2 (samples 1–3), where we believe they are mainly nonphosphorylated. Even under these relaxing conditions, some thick filaments appeared to have thin filaments attached to them.

The filaments were tested for any direct effect of Ca^{2+} on their structure by rinsing for 5–10 s with 6–10 drops of activating rinse (relaxing rinse + 10^{-4} M free $[\text{Ca}^{2+}]$) immediately before staining (cf., Vibert and Craig, 1985). Filaments showed a similar ordered, helical structure to that seen at low Ca^{2+} levels (Fig. 1 c).

Thick Filaments in Activating Medium

Filament suspensions were incubated at 25°C in activating medium (containing 10^{-4} M free Ca^{2+}) and after 5, 10, and 30 min, aliquots were removed for determining the extent of light chain phosphorylation and simultaneously for examining thick filament structure by negative staining. Grids were washed with activating rinse immediately before staining. From the earliest time point (5 min), thick filaments incubated in activating medium had completely phosphorylated light chains (Fig. 2, samples 7–9). They also looked clearly different in structure from those in relaxing medium: the ordered helical structure of the thick filaments was generally lost (Fig. 3) and the crossbridges sometimes appeared clumped or to project further from the filament backbone (Fig. 3 c).

The disorder was not due to the slight (0.15 U) drop in pH that occurred on addition of Ca^{2+} to the relaxing medium, since relaxed filaments rinsed with relaxing rinse at this lower pH still showed helical crossbridge order. The disorder produced by activation was not reversed by rinsing with relaxing (low Ca^{2+}) rinse before staining (Fig. 3 c).

To prevent any reversal of phosphorylation by endogenous phosphatase, filaments were also incubated in activating

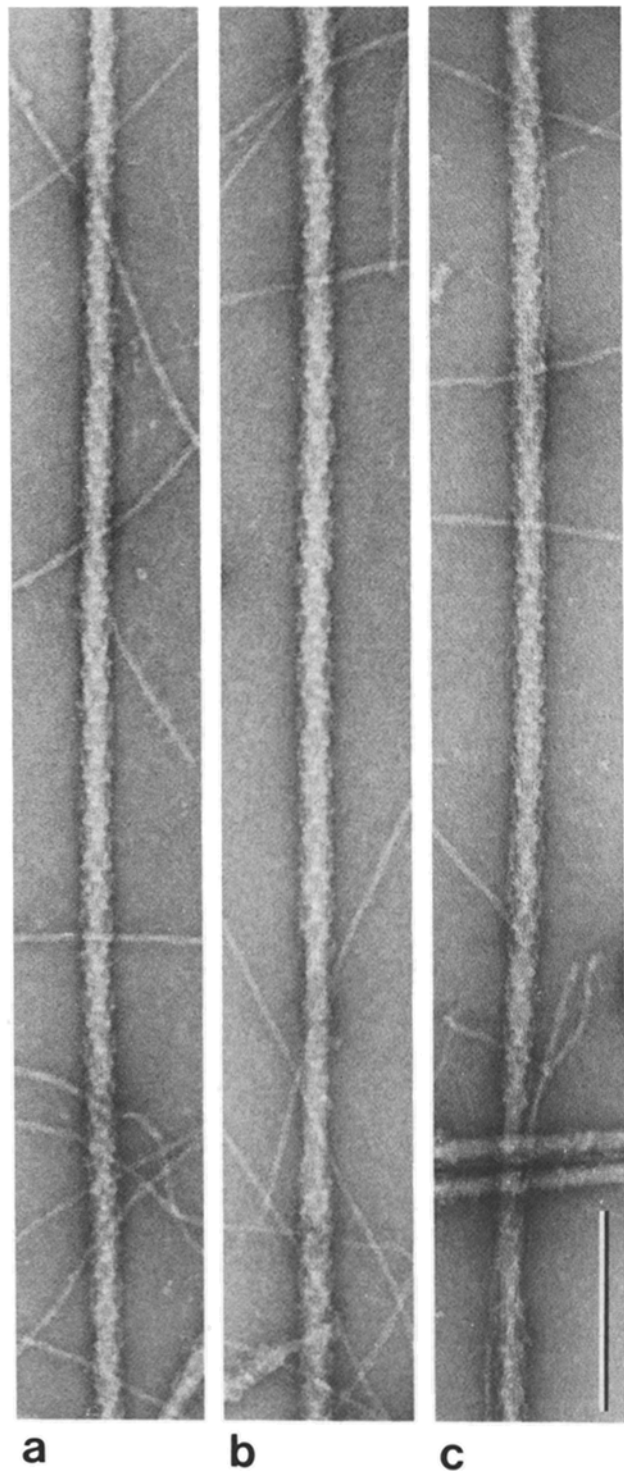


Figure 1. Relaxed tarantula thick filaments (light chains non-phosphorylated) negatively stained with 1% uranyl acetate. Filaments were incubated in relaxing medium for 10 (a), 30 (b), and 15 (c) min before rinsing on the grid with relaxing rinse (a and b) or activating rinse (c). The appearance of helical order is enhanced by viewing the filaments obliquely along their length or by turning them horizontally. In all electron micrographs in this and other figures the bare zone of the filament (not always visible) is towards the bottom. Bar, 0.2 μm .

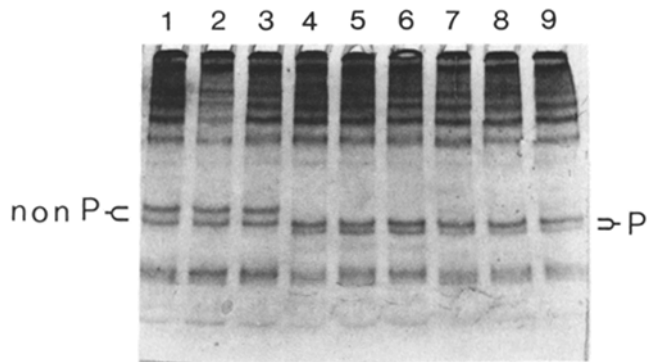


Figure 2. Light chain phosphorylation of tarantula filament suspensions monitored by electrophoresis on 10% acrylamide–40% glycerol/Tris-glycine buffer gels. Filament suspensions were incubated at 25°C in relaxing medium (MgATP, EGTA) for 5, 10, and 30 min (samples 1, 2, and 3); in relaxing medium plus 50 µg/ml trypsin-treated (Ca^{2+} -insensitive) MLCK for 5, 10, and 30 min (samples 4, 5, and 6); and in activating medium (0.1 mM free $[\text{Ca}^{2+}]$) for 5, 10, and 30 min (samples 7, 8, and 9). Aliquots of the filament suspensions were taken for electron microscopy at the same time points as for electrophoresis. Gel samples were prepared rapidly and the gels run as soon as possible as described in Materials and Methods (to prevent any possible change that may occur on storage). Shown is a typical dried Coomassie-stained gel run immediately at the end of the experiment. *Non P* denotes the positions of the regulatory light chains in relaxing medium (presumed to be mainly nonphosphorylated), and *P* marks their position after incubation in activation buffer and endogenous kinase or with Ca^{2+} -insensitive kinase (phosphorylated).

medium containing ATP- γ -S instead of ATP. A similar degree of disorder of the crossbridge array was observed to that seen with normal activating medium and the glycerol/urea gel patterns were also similar.

Attempts to reverse the phosphorylation, by addition of a phosphatase isolated from chicken gizzard muscle (Kendrick-Jones et al., 1983) were unsuccessful as judged by urea/glycerol gel electrophoresis and electron microscopy: only a small amount of light chain dephosphorylation was observed and no significant decrease in crossbridge disorder was seen. In addition, prolonged incubation of the filament preparations, even in EGTA, slowly renders the kinase Ca^{2+} insensitive, presumably due to proteolytic cleavage (Adelstein et al., 1981), and thus leads to a steady increase in kinase activity and to a decrease in dephosphorylation.

Thin filaments were sometimes observed attached to thick filaments under activating conditions. Crossbridges at various angles appeared to link thick and thin filaments (Fig. 4), possibly representing interactions between actively sliding filaments that were trapped by uranyl acetate staining.

Thick Filaments in Relaxing Medium Containing Desensitized Myosin Light Chain Kinase

The regulatory light chains can also be phosphorylated in relaxing medium, i.e., at low Ca^{2+} levels, by the addition of trypsin-treated gizzard MLCK, which does not require Ca^{2+} for its activity (Fig. 2, samples 4–6). Filaments phosphorylated at low $[\text{Ca}^{2+}]$ in this way were applied to a grid and washed with relaxing rinse. They showed loss of helical ordering of the crossbridges not obviously distinguishable

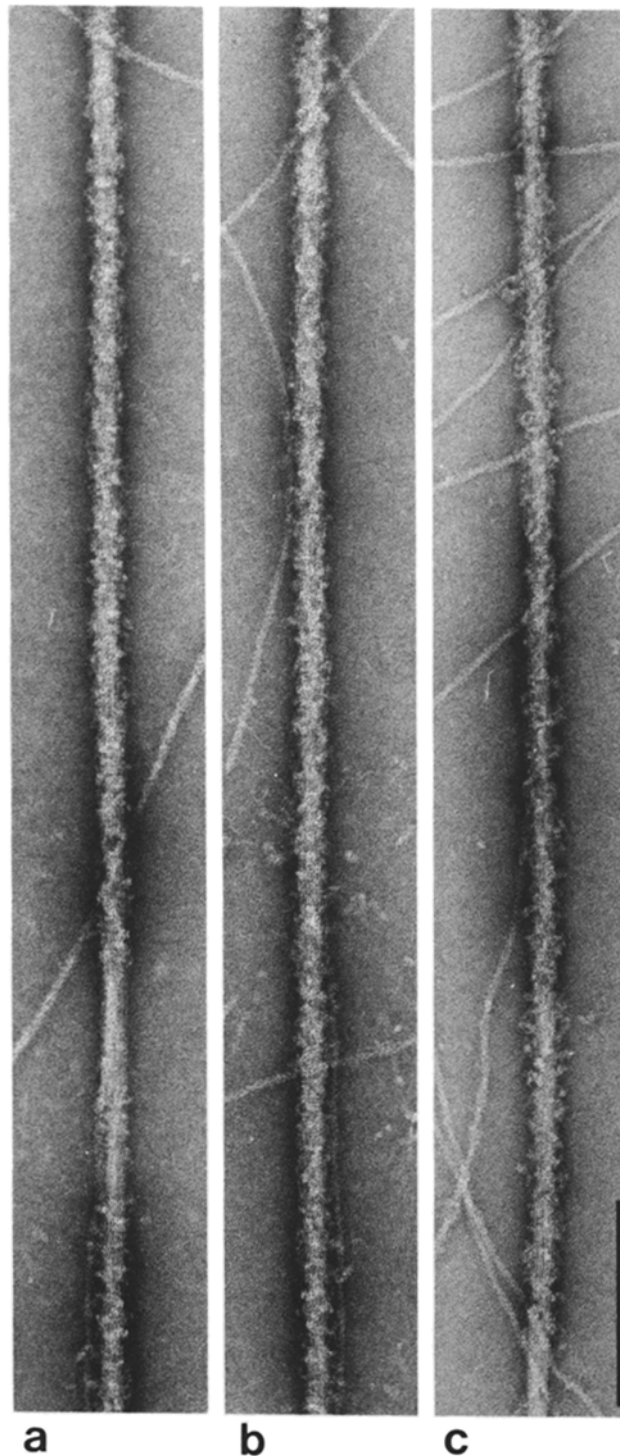
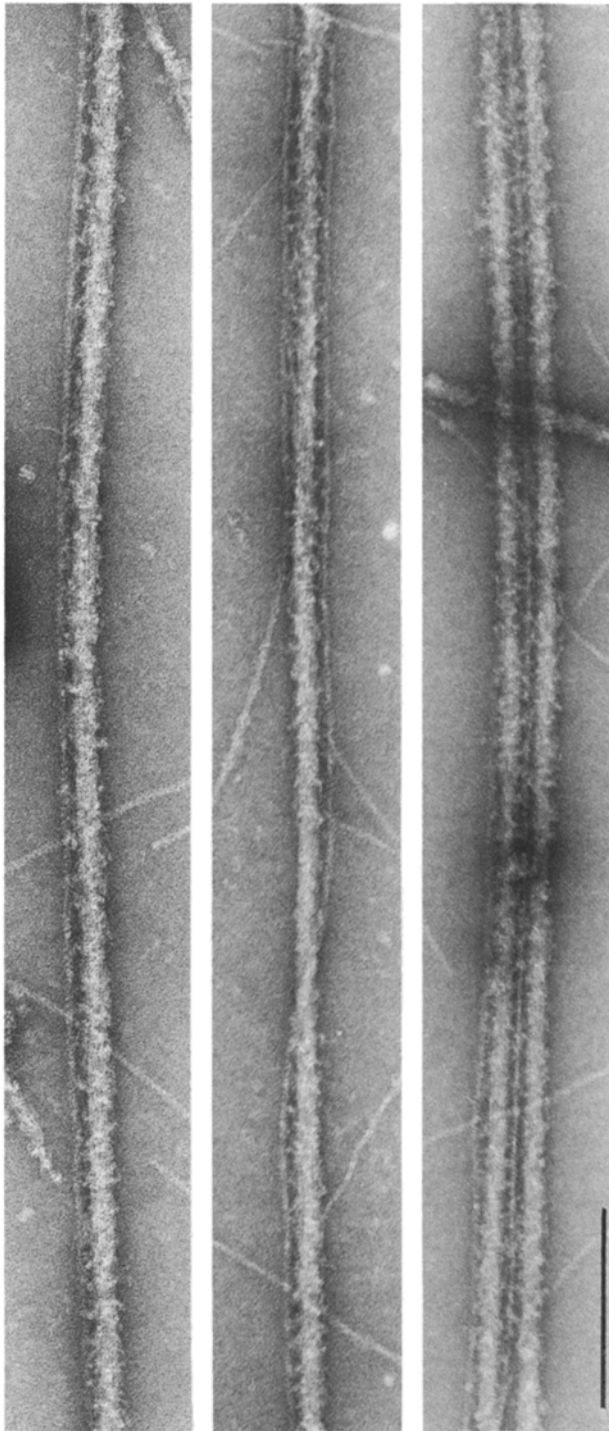


Figure 3. Negatively stained tarantula thick filaments after Ca^{2+} -induced phosphorylation by endogenous MLCK and calmodulin. Grids were rinsed with activating rinse (a and b) or relaxing rinse (c) before staining. Bar, 0.2 µm.

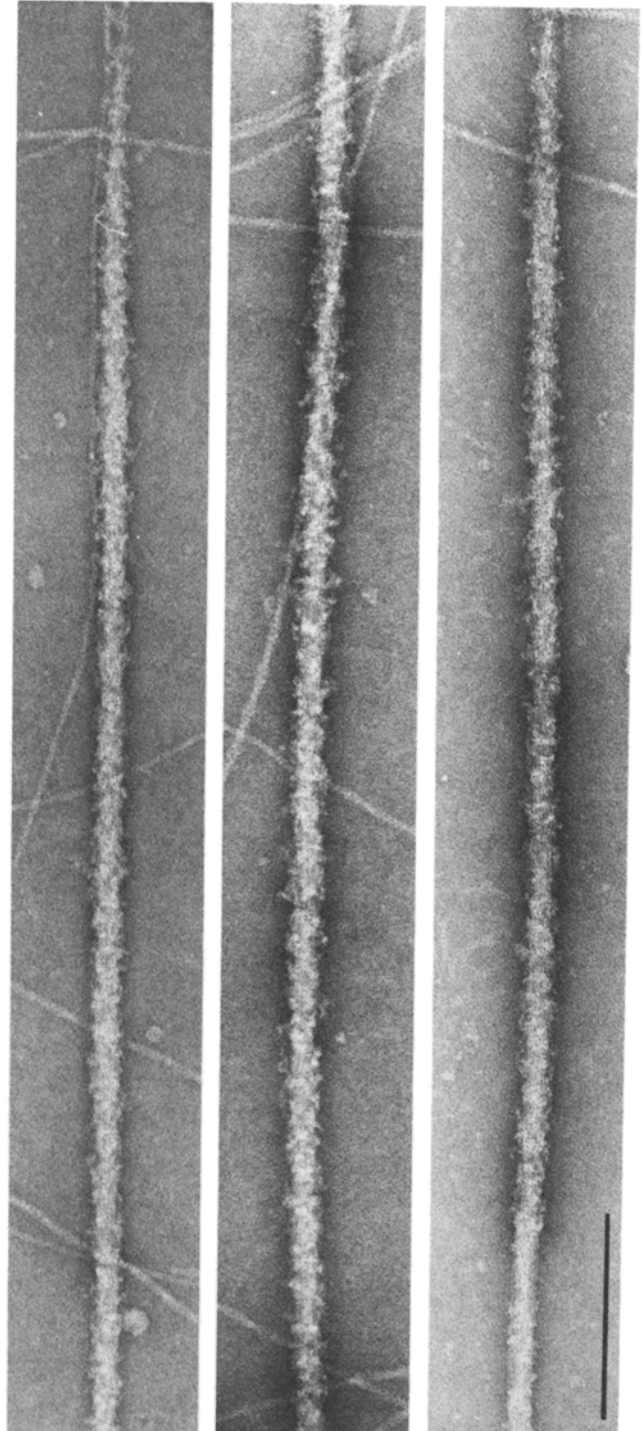
from that occurring at high $[\text{Ca}^{2+}]$ (Fig. 5). The structure was not obviously altered by subsequent exposure to high $[\text{Ca}^{2+}]$ (rinsing with activating rinse; Fig. 5 c). These results suggest that phosphorylation of the regulatory chains, rather than $[\text{Ca}^{2+}]$ per se, is the factor responsible for disordering of the crossbridge array.



a **b** **c**
Figure 4. Negatively stained filament homogenate in activating conditions showing crossbridges between thick and thin filaments. Bar, 0.2 μm .

Biochemical Studies of Tarantula Thick Filaments

To establish the polypeptide composition of tarantula thick filaments and to demonstrate that the changes observed on the urea/glycerol gels are due to myosin light chain phosphorylation, tarantula myosin was isolated and purified. Gel analysis of the purified myosin and of the crude filament



a **b** **c**
Figure 5. Negatively stained tarantula thick filaments phosphorylated at low Ca^{2+} by the addition of Ca^{2+} -insensitive gizzard myosin light chain kinase. Grids were rinsed with relaxing rinse (*a* and *b*) or activating rinse (*c*) before staining. Bar, 0.2 μm .

homogenates used for electron microscopy revealed the following. (*a*) The myosin contains two types of light chains of 26 and 19 kD (Fig. 6 C); corresponding bands are seen on SDS gels of the filament homogenates (Fig. 6 E). (*b*) In both the purified myosin and the filament homogenates, only the

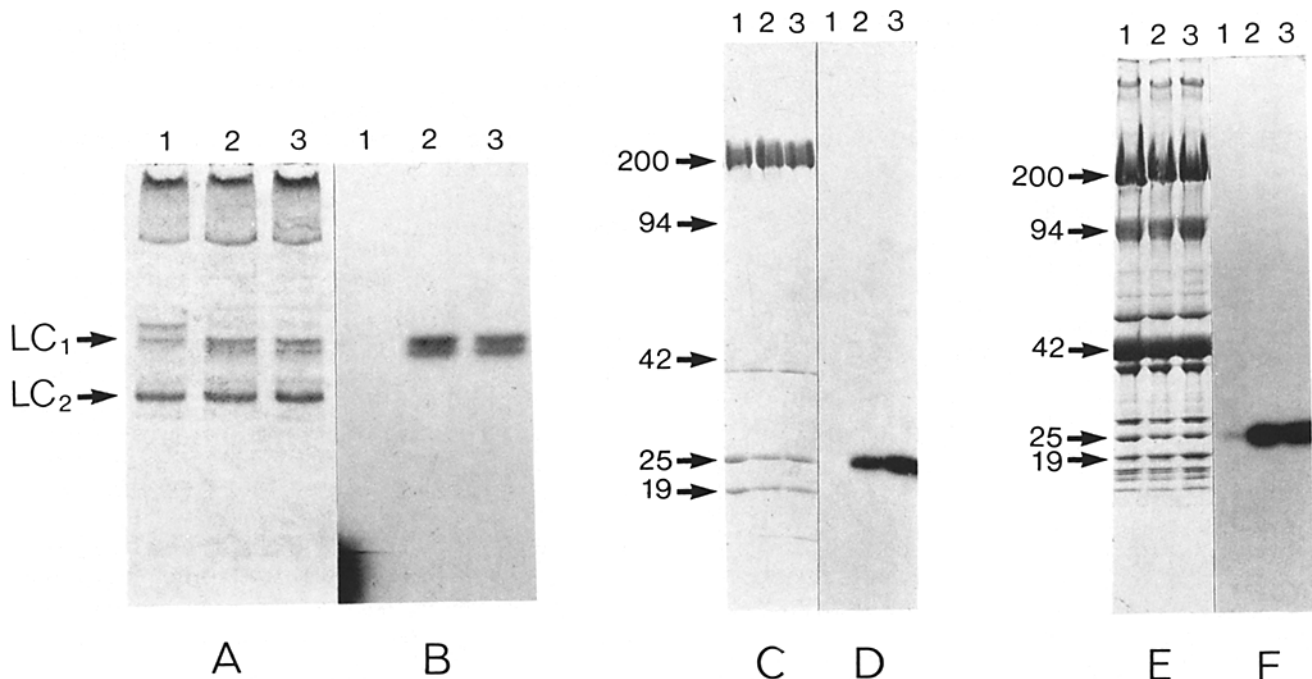


Figure 6. Gel electrophoresis of tarantula myosin and filament suspensions demonstrating that the phosphorylatable bands are the regulatory light chains of 26 kD (LC_1). (A) Coomassie-stained urea/glycerol PAGE of tarantula myosin incubated in 150 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 1 mM DTT, and 2.5 mM [^{32}P]- γ -ATP (5 $\mu Ci/\mu mol$) at 25°C; sample 1 incubated in 0.1 mM EGTA and 25 $\mu g/ml$ gizzard MLCK for 15 min, and samples 2 and 3 incubated in 0.1 mM Ca^{2+} and 25 $\mu g/ml$ MLCK for 2 and 5 min. (In this experiment calmodulin was not added: traces were present, even after exhaustive purification, that were sufficient to activate the MLCK.) (B) Autoradiogram of gel A. The two bands in the position of the regulatory light chains (labeled LC_1) are rapidly phosphorylated at about the same rate and incorporate about the same amount of [^{32}P]phosphate. This gel further demonstrates that the change in mobility of the light chains is associated with [^{32}P]phosphate incorporation. LC_2 denotes the "essential" light chains. (C) Coomassie-stained SDS gel (5–20% acrylamide gradient) of tarantula myosin incubated under exactly the same conditions as in gel A; i.e., sample 1 in EGTA and MLCK for 15 min, samples 2 and 3 in Ca^{2+} + MLCK for 2 and 5 min, respectively. (D) Autoradiogram of gel C. These gels were deliberately overloaded, which caused "smearing" of the myosin heavy chain, to demonstrate that only the 26-kD light chain (LC_1) incorporated [^{32}P]phosphate, which was maximal by 5 min (no further [^{32}P]phosphate was incorporated even when incubated for up to 30 min). (E) Coomassie-stained SDS gel (5–20% acrylamide gradient) of a tarantula filament suspension incubated in a medium containing [^{32}P]- γ -ATP (5 $\mu Ci/\mu mol$) at 25°C; sample 1 in relaxing medium (EGTA/ATP) for 35 min; samples 2 and 3 in relaxing medium + Ca^{2+} -insensitive MLCK for 5 and 10 min, respectively. (F) Autoradiogram of gel E. These gels have again been overloaded, which causes "smearing" of the myosin heavy chain and actin bands, to further demonstrate that in these crude filament suspensions only the 26-kD light chain (LC_1) incorporates [^{32}P]phosphate, and that this incorporation, which is rapid, occurs in relaxing medium only in the presence of Ca^{2+} -insensitive MLCK. Numbers at sides of gels indicate the positions (in kD) at which molecular mass markers described in Materials and Methods run.

regulatory (26 kD) light chain is phosphorylated by the endogenous and the purified gizzard kinases (Fig. 6, C–F). (c) Purified myosin preparations run on glycerol/urea gel electrophoresis showed two bands in the expected position of the regulatory light chain (Fig. 6 A). When the myosin was phosphorylated, the upper band disappeared and a new band appeared with higher mobility than the lower one (Fig. 6 A); the latter two bands both incorporated [^{32}P]phosphate (Fig. 6 B). Filament homogenates gave exactly the same result (Figs. 2 and 6 E). We presume, therefore, that these bands must be the regulatory light chains and that the change in mobility must be due to phosphorylation. The two bands are present in a roughly constant ratio in the filament and myosin preparations and their extent of [^{32}P]phosphate incorporation is about the same (Fig. 6 B). The simplest interpretation of these results is that the light chains are originally non-phosphorylated and that both become singly phosphorylated at about the same rate. Thus, tarantula myosin appears to

contain two phosphorylatable regulatory light chain species which differ slightly in net charge, but have the same molecular weight (26 kD). This is different from the situation in *Limulus* striated muscle (Sellers, 1981).

The interaction of the purified tarantula myosin with purified rabbit actin, as measured by the actin-activated myosin MgATPase activity, did not appear to be controlled by Ca^{2+} , but phosphorylation caused a significant potentiation of the ATPase activity (Table I).

Discussion

In this paper we have shown that phosphorylation of the regulatory light chains modulates the actin activation of tarantula myosin ATPase and that paralleling this there is a change in the arrangement of the myosin crossbridges from a helically ordered to a disordered state.

Table I. Effect of Phosphorylation on the Actin-activated MgATPase Activity of Tarantula Myosin (AM-MgATPase)

Tarantula Myosin	AM-MgATPase	
	-Ca ²⁺	+Ca ²⁺
	<i>μmol H⁺ · min⁻¹ per · myosin⁻¹</i>	
Nonphosphorylated myosin alone	0.012 ± 0.005	0.021 ± 0.010
Nonphosphorylated myosin + actin-tropomyosin	0.226 ± 0.031	0.276 ± 0.037
Thiophosphorylated myosin alone	0.017 ± 0.009	0.052 ± 0.021
Thiophosphorylated myosin + actin-tropomyosin	0.576 ± 0.068	0.585 ± 0.048

Assays were carried out in a pH-stat at pH 7.6 and 25°C at a final concentration (final volume 10 ml) of 48 mM NaCl, 5 mM MgCl₂, 2 mM MgATP, and either 0.1 mM EGTA (-Ca²⁺) or 0.1 mM EGTA + 0.15 mM CaCl₂ (+Ca²⁺). Nonphosphorylated (i.e., the myosin as prepared) or thiophosphorylated myosin (0.5 mg) were mixed with rabbit F-actin (1 mg) and rabbit tropomyosin (0.4 mg) in a final vol of 200 μl of 0.6 M NaCl, 5 mM phosphate, pH 7.0, 1 mM MgATP, and the whole sample was taken for assay. Tropomyosin was included in the assay because it had previously been shown to potentiate the actin-activated MgATPase of *Limulus* myosin (Lehman and Szent-Györgyi, 1972).

The results shown are the means and standard deviations obtained with three different myosin preparations, including one preparation where the myosin was subjected to further purification by gel filtration (Sephacrose 4B-C1 chromatography). As a further control, the phosphorylation level of the nonphosphorylated myosin samples after the ATPase assays were tested and found not to be changed.

Effect of Light Chain Phosphorylation on Tarantula Myosin

Tarantula thick filaments contain phosphorylatable regulatory light chains of 26 kD. Under relaxing conditions these light chains appear to be nonphosphorylated, and become rapidly phosphorylated when Ca²⁺ is added to activate the endogenous kinase or when trypsin-treated gizzard kinase is added in the absence of Ca²⁺ (Figs. 2 and 6).

The ATPase results suggest that in our myosin preparations, light chain phosphorylation is not an essential requirement for the actin-activated myosin MgATPase activity, although it does have a pronounced potentiating effect (Table I). This situation is similar to that in vertebrate striated muscles where, although light chain phosphorylation occurs both in vivo and in vitro, there is no direct evidence that it is involved in regulating myosin interaction with actin (Morgan et al., 1976; Manning and Stull, 1979; Pemrick, 1980; Barsotti and Butler, 1984). However, it may, for example, potentiate the force at submaximal Ca²⁺ levels (Persechini et al., 1985). The situation is different from vertebrate smooth muscle, where light chain phosphorylation is essential for myosin regulation (Adelstein and Eisenberg, 1980).

Sellers (1981) demonstrated that myosin isolated from the striated (telson) muscles of *Limulus* (the horseshoe crab), a closely related arthropod, is regulated by light chain phosphorylation. This raises the possibility that the lack of such an effect in tarantula myosin is due to some "modification" of the myosin during preparation. Although we cannot exclude this possibility, we think that it is unlikely for the following reasons. (a) Four different myosin preparations gave basically the same results. (b) The myosin preparative procedure is extremely rapid and does not use extreme conditions. DTT and proteolytic inhibitors were included and there was no sign of proteolytic degradation of either heavy chain or

light chain. Gizzard myosin was prepared by the same procedure in parallel, and was shown to be regulated by light chain phosphorylation. (c) The light chains appear to be mainly nonphosphorylated when the myosins are prepared and can be phosphorylated to greater than 90% by endogenous kinase or gizzard MLCK. Myosins prepared by the same or similar procedures (e.g., *Limulus* myosin [Sellers, 1981], thymus myosin [Scholey et al., 1982], gizzard myosin [Kendrick-Jones et al., 1983], rabbit skeletal muscle myosin [Kendrick-Jones, J., unpublished observations]) are all nonphosphorylated (>90%) when prepared. (d) The myosins have actin-activated MgATPase activities which are in the expected range for a striated muscle myosin (cf., vertebrate striated muscle myosin; Morgan et al., 1976) and which are remarkably consistent between the different myosin preparations. (e) The myofibrils at the initial stage of myosin preparation are Ca²⁺ regulated and therefore one presumes the regulatory proteins are intact and unmodified.

We therefore conclude that in tarantula striated muscle, myosin light chain phosphorylation is not the "primary" regulatory mechanism, but may play a role in facilitating myosin crossbridge interaction with actin.

Structural Changes Associated with Phosphorylation of the Myosin Light Chains

Relaxed (nonphosphorylated) tarantula filaments have a well-ordered helical array of crossbridges (Fig. 1) and show strong first and fourth layer lines and third and sixth order meridional reflections, indexing on a repeat of 43 nm, by optical diffraction (Crowther et al., 1985; Levine et al., 1983). The similarity of these patterns to X-ray diffraction patterns of whole relaxed tarantula muscle (Wray, 1982) suggests that the native relaxed state is well preserved by negative staining and that these filaments provide a good basis for observing any structural changes that may be induced by phosphorylation.

When relaxed filaments were incubated in activating medium, the regulatory light chains were rapidly phosphorylated and concomitantly the thick filaments equally rapidly lost their crossbridge order. There was no obvious shortening or dissolution of the myosin filaments on phosphorylation as might have been expected by comparison with studies of *Limulus* filaments (Brann et al., 1979; Levine and Kensler, 1985; Levine, 1986). Although we were unable to reverse phosphorylation to any significant extent owing to the lack of a purified phosphatase having a sufficiently high activity to overcome the high level of endogenous myosin kinase activity in these filament preparations, we believe that the disordering we observed is a specific result of phosphorylation for the following reasons. (a) The structural changes correlate in time with light chain phosphorylation induced by Ca²⁺. (b) Light chain phosphorylation induced at low Ca²⁺ concentrations by the addition of a Ca²⁺-insensitive kinase also correlates with a transition to disorder of the crossbridge array (Fig. 5). Thus Ca²⁺ is unnecessary for the disordering and Ca²⁺-activated proteases are also unlikely to be its cause. Since tarantula muscle appears to have actin-linked regulation (data not shown; see also Lehman and Szent-Györgyi, 1975), the changes observed at low Ca²⁺ also imply that crossbridge disordering is not simply a result of the cyclic interactions of crossbridges with actin that

may occur in filament homogenates in high Ca^{2+} (activating) conditions. The gradual phosphorylation that occurs when a filament preparation stands in low Ca^{2+} (relaxing) medium for several hours probably results from a gradual loss of Ca^{2+} sensitivity (and thus activation) of endogenous kinase and is probably responsible for the gradual loss of helical order of filaments left to stand for such periods. (c) Disorder is not a direct result of Ca^{2+} binding since rinsing a relaxed preparation of filaments on the grid with activating rinse did not alter the structure of the filaments (cf., scallop filaments, which are disordered by such a Ca^{2+} rinse; Vibert and Craig, 1985). Similar rinsing of a preparation which had first been phosphorylated at low Ca^{2+} caused no further loss of order.

Loss of relaxed order has been observed in other species of myosin filaments when they are activated. Levine (1986) has recently reported a loss of helical order in *Limulus* myosin filaments when the regulatory light chains are phosphorylated. Filament bundles formed from purified vertebrate smooth muscle myosin lose their crossbridge periodicity when they are phosphorylated (Ikebe and Ogihara, 1982). In scallop filaments, activation occurs not by phosphorylation but by direct binding of Ca^{2+} to the myosin heads. The periodic arrangement of crossbridges characteristic of relaxed filaments is lost on exposure to activating levels of Ca^{2+} (Vibert and Craig, 1985). These observations suggest that, although the regulatory switch may be either binding of Ca^{2+} to the myosin heads or phosphorylation of the regulatory light chains in the myosin heads, the structural effects are similar.

The structural differences we observe between relaxed and phosphorylated filaments suggest that the crossbridges are held in a well ordered array in relaxed filaments by interactions between heads of neighboring molecules (Crowther et al., 1985) or by binding of the heads to the backbone, and that when the filaments are phosphorylated these interactions are weakened so that the crossbridges are free to adopt a wide variety of configurations. Equatorial X-ray diffraction patterns of demembrated tarantula muscle are consistent with an average 6-nm movement of the crossbridges away from the filament backbone after thiophosphorylation (Sosa, 1986; Sosa et al., 1986a, b; Panté, 1986; Panté et al., 1986a, b), further supporting the view that the crossbridges are more weakly bound to the filament in the phosphorylated state. In the intact muscle, this loosening may facilitate the interaction of crossbridges with actin filaments during contraction.

In the scallop, removal of the regulatory light chains also causes loss of crossbridge order, suggesting that these light chains are essential in binding the heads to the filament (Vibert and Craig, 1985). If the light chains also have such a role in tarantula, then it is not surprising that phosphorylation of this critical region of the myosin head can have such a potent effect on crossbridge binding to the filament.

The binding of myosin heads to the filament also appears to be weakened by increased salt concentration (Wray et al., 1974; Levine et al., 1986), lowering of ATP or Mg^{2+} levels (Haselgrove, 1975; Barnett and Thomas, 1984; Persechini and Rowe, 1984; Pinset-Härström, 1985; Vibert and Craig, 1985; Levine et al., 1986), and by changes in pH (Levine et al., 1986) suggesting that ionic interactions are important in stabilizing the crossbridge array and that small changes in

the charge on the crossbridges are sufficient to release them from the filament backbone.

Related changes in structure have been observed in isolated myosin molecules from vertebrate smooth and nonmuscle cells. In low salt conditions (in the presence of ATP and Mg^{2+} , and with light chains dephosphorylated), these molecules generally have a folded structure, where the myosin tail bends back up and binds to the heads and the heads point down towards the tail (Trybus et al., 1982; Onishi and Wakabayashi, 1982; Craig et al., 1983). If the salt level is increased, the light chains are phosphorylated, or ATP is removed, the equilibrium moves in the direction of tail extension and random orientations of the heads (Onishi et al., 1983; Craig et al., 1983; Trybus and Lowey, 1984; Suzuki et al., 1985, 1986). These changes are analogous to those we have observed if we assume that in the filaments, the heads of myosin molecules are bound to the backbone through interaction with tails of different molecules (due to the staggered arrangement of molecules in the filament shaft), while in isolated molecules the interactions are intramolecular.

We would like to dedicate this paper to the memory of Marcus Kress.

We thank Drs. H. E. Huxley and R. A. Crowther for discussion and Ms. A. Trischitta and Ms. M. Giorgio for photographic assistance.

This work was supported by grants from the National Institutes of Health (AM-34711) and the Muscular Dystrophy Association to R. Craig, and from the Muscular Dystrophy Association and CONICIT (SI-1460) to R. Padrón.

Received for publication 30 December 1986, and in revised form 5 May 1987.

References

- Adelstein, R. S., and E. Eisenberg. 1980. Regulation and kinetics of the actin-myosin-ATP interaction. *Annu. Rev. Biochem.* 49:921-956.
- Adelstein, R. S., and C. B. Klee. 1981. Purification and characterization of smooth muscle myosin light chain kinase. *J. Biol. Chem.* 256:7501-7509.
- Adelstein, R. S., M. D. Pato, J. R. Sellers, P. de Lanerolle, and M. A. Conti. 1981. Regulation of actin-myosin interaction by reversible phosphorylation of myosin and myosin kinase. *Cold Spring Harbor Symp. Quant. Biol.* 46: 921-928.
- Bailey, K. 1948. Tropomyosin: a new asymmetric protein component of the muscle fibril. *Biochem. J.* 43:271-279.
- Barnett, V. A., and D. D. Thomas. 1984. Saturation transfer electron paramagnetic resonance of spin-labeled muscle fibers: dependence of myosin head rotational motion on sarcomere length. *J. Mol. Biol.* 179:83-102.
- Barsotti, R. J., and T. M. Butler. 1984. Chemical energy usage and myosin light chain phosphorylation in mammalian skeletal muscle. *J. Muscle Res. Cell Motil.* 5:45-64.
- Brann, L., M. M. Dewey, E. A. Baldwin, P. Brink, and B. Walcott. 1979. Requirements for *in vitro* shortening and lengthening of isolated thick filaments of *Limulus* striated muscle. *Nature (Lond.)* 279:256-257.
- Craig, R., R. Padrón, and J. Kendrick-Jones. 1985. Structural changes accompanying phosphorylation of tarantula muscle thick filaments. *Biophys. J.* 47:469a. (Abstr.)
- Craig, R., R. C. Smith, and J. Kendrick-Jones. 1983. Light chain phosphorylation controls the conformation of vertebrate non-muscle and smooth muscle myosin molecules. *Nature (Lond.)* 302:436-439.
- Crowther, R. A., R. Padrón, and R. Craig. 1985. Arrangement of the heads of myosin in relaxed thick filaments from tarantula muscle. *J. Mol. Biol.* 184:429-439.
- Feuer, G., F. Molnar, E. Pettko, and F. B. Straub. 1948. *Hung. Acta Physiol.* 1:150-160.
- Focant, B., and F. Hurliaux. 1976. Light chains of carp and pike skeletal muscle myosins. Isolation and characterization of the most anodic light chain on alkaline pH electrophoresis. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 65:16-20.
- Greaser, M. L., and J. Gergely. 1971. Reconstitution of troponin activity from three protein components. *J. Biol. Chem.* 246:4226-4233.
- Haselgrove, J. C. 1975. X-ray evidence for conformational changes in the myosin filaments of vertebrate striated muscle. *J. Mol. Biol.* 92:113-143.
- Ikebe, M., and Ogihara, S. 1982. Phosphorylation-dependent and ATP-induced changes in structural array in gizzard myosin filament bundles. *J. Biochem. (Tokyo)* 92:1973-1977.
- Kendrick-Jones, J., and J. M. Scholey. 1981. Myosin-linked regulatory systems. *J. Muscle Res. Cell Motil.* 2:347-372.

- Kendrick-Jones, J., W. Z. Cande, P. J. Tooth, R. C. Smith, and J. M. Scholey. 1983. Studies on the effect of phosphorylation of the 20,000 *M*₁ light chain of vertebrate smooth muscle myosin. *J. Mol. Biol.* 165:139-162.
- Kendrick-Jones, J., W. Lehman, and A. G. Szent-Györgyi. 1970. Regulation in molluscan muscles. *J. Mol. Biol.* 54:313-326.
- Kendrick-Jones, J., E. M. Szent-Kiralyi, and A. G. Szent-Györgyi. 1976. Regulatory light chains in myosins. *J. Mol. Biol.* 104:747-775.
- Kensler, R. W., and R. J. C. Levine. 1982. An electron microscopic and optical diffraction analysis of the structure of *Limulus* telson muscle thick filaments. *J. Cell Biol.* 92:443-451.
- Lehman, W., and A. G. Szent-Györgyi. 1972. Activation of the adenosine triphosphatase of *Limulus polyphemus* actomyosin by tropomyosin. *J. Gen. Physiol.* 59:375-387.
- Lehman, W., and A. G. Szent-Györgyi. 1975. Regulation of muscular contraction. Distribution of actin control and myosin control in the animal kingdom. *J. Gen. Physiol.* 66:1-30.
- Levine, R. J. C. 1986. Effects of myosin light chain phosphorylation on *Limulus* thick filaments. *J. Cell Biol.* 103(5, Pt. 2):118a. (Abstr.)
- Levine, R. J. C., and R. W. Kensler. 1985. Structure of short thick filaments from *Limulus* muscle. *J. Mol. Biol.* 182:347-352.
- Levine, R. J. C., R. W. Kensler, and P. Levitt. 1986. Crossbridge and backbone structure of invertebrate thick filaments. *Biophys. J.* 49:135-138.
- Levine, R. J. C., R. W. Kensler, M. C. Reedy, W. Hofmann, and H. A. King. 1983. Structure and paramyosin content of tarantula thick filaments. *J. Cell Biol.* 97:186-195.
- Manning, D. R., and J. T. Stull. 1979. Myosin light chain phosphorylation and phosphorylase A activity in rat extensor digitorum longus muscle. *Biochem. Biophys. Res. Commun.* 90:164-170.
- Matsudaira, P. T., and D. R. Burgess. 1978. SDS microslab linear gradient polyacrylamide gel electrophoresis. *Anal. Biochem.* 87:386-396.
- Morgan, M., S. V. Perry, and J. Ottaway. 1976. Myosin light chain phosphatase. *Biochem. J.* 157:687-697.
- Onishi, H., and T. Wakabayashi. 1982. Electron microscopic studies of myosin molecules from chicken gizzard muscle. I. The formation of the intramolecular loop in the myosin tail. *J. Biochem. (Tokyo)*. 92:871-879.
- Onishi, H., T. Wakabayashi, T. Kamata, and S. Watanabe. 1983. Electron microscopic studies of myosin molecules from chicken gizzard muscle. II. The effect of thiophosphorylation of the 20k-Dalton light chain on the ATP-induced change in the conformation of myosin monomers. *J. Biochem. (Tokyo)*. 94:1147-1154.
- Padrón, R., R. A. Crowther, and R. Craig. 1984. Three-dimensional reconstruction of native tarantula muscle thick filaments. *Biophys. J.* 45:10a. (Abstr.)
- Panté, N., H. Sosa, and R. Padrón. 1986a. Prediction of relative changes in the equatorial x-ray diffraction pattern of striated muscle after activation. *Acta Cient. Venez.* 37:223-225.
- Panté, N., H. Sosa, and R. Padrón. 1986b. Study by x-ray diffraction of structural changes after phosphorylation of tarantula muscle thick filaments. *Acta. Cient. Venez.* 37(1):61. (Abstr.)
- Panté, N. 1986. Estudio por difracción de rayos-X de los cambios estructurales causados por la activación de la contracción muscular. M.Sc. Thesis, Centro de Estudios Avanzados (CEA), Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas, Venezuela.
- Pemrick, S. M. 1980. The phosphorylated *L*₂ light chain of skeletal myosin is a modifier of the actomyosin ATPase. *J. Biol. Chem.* 255:8836-8841.
- Perrie, W. T., and S. V. Perry. 1970. An electrophoretic study of the low-molecular-weight components of myosin. *Biochem. J.* 119:31-38.
- Persechini, A., and A. J. Rowe. 1984. Modulation of myosin filament conformation by physiological levels of divalent cation. *J. Mol. Biol.* 172:23-39.
- Persechini, A., J. T. Stull, and R. Cooke. 1985. The effect of myosin phosphorylation on the contractile properties of skinned rabbit skeletal muscle fibers. *J. Biol. Chem.* 260:7951-7954.
- Pinset-Härström, I. 1985. Mg ATP specifically controls *in vitro* self-assembly of the vertebrate skeletal myosin in the physiological pH range. *J. Mol. Biol.* 182:159-172.
- Scholey, J. M., R. C. Smith, D. Drenckhahn, U. Groschel-Stewart, and J. Kendrick-Jones. 1982. Thymus Myosin. Isolation and characterization of myosin from calf thymus and thymic lymphocytes, and studies on the effect of phosphorylation of its *M*₁ = 20,000 light chain. *J. Biol. Chem.* 257:7737-7745.
- Scholey, J. M., K. A. Taylor, and J. Kendrick-Jones. 1981. The role of myosin light chains in regulating actin-myosin interaction. *Biochimie.* 63:255-271.
- Sellers, J. R. 1981. Phosphorylation-dependent regulation of *Limulus* myosin. *J. Biol. Chem.* 256:9274-9278.
- Small, J. V., and A. Sobieszek. 1980. The contractile apparatus of smooth muscle. *Int. Rev. Cytol.* 64:241-306.
- Sosa, H. 1986. Estudio por difracción de rayos-X de la estructura transversal del sarcomero de musculo estriado de tarantula en diferentes condiciones experimentales. Tesis Lic. Biol., Universidad Central de Venezuela (UCV), Caracas, Venezuela.
- Sosa, H., N. Panté, and R. Padrón. 1986a. Recording of the equatorial section of the x-ray diffraction pattern of tarantula live muscle. *Acta Cient. Venez.* 37:587-588.
- Sosa, H., N. Panté, and R. Padrón. 1986b. Sarcomere transverse structure of tarantula skinned muscle in relaxed or rigor states. *Acta Cient. Venez.* 37(1):10. (Abstr.)
- Suzuki, H., Y. Kondo, A. D. Carlos, and J. C. Seidel. 1986. A conformational transition in gizzard heavy meromyosin involving the head-tail junction studied by rates of digestion of heavy and light chains by papain. *Biophys. J.* 49:184a. (Abstr.)
- Suzuki, H., W. F. Stafford, H. S. Slayter, and J. C. Seidel. 1985. A conformation transition in gizzard heavy meromyosin involving the head-tail junction, resulting in changes in sedimentation coefficient, ATPase activity, and orientation of heads. *J. Biol. Chem.* 260:14810-14817.
- Szent-Györgyi, A. G., E. M. Szent-Kiralyi, and J. Kendrick-Jones. 1973. The light chains of scallop myosin as regulatory subunits. *J. Mol. Biol.* 74:179-203.
- Trybus, K. M., and S. Lowey. 1984. Conformational states of smooth muscle myosin. *J. Biol. Chem.* 259:8564-8571.
- Trybus, K. M., T. W. Huiatt, and S. Lowey. 1982. A bent monomeric conformation of myosin from smooth muscle. *Proc. Natl. Acad. Sci. USA.* 79:6151-6155.
- Vibert, P., and R. Craig. 1982. Three-dimensional reconstruction of thin filaments decorated with a Ca²⁺-regulated myosin. *J. Mol. Biol.* 157:299-319.
- Vibert, P., and R. Craig. 1983. Electron microscopy and image analysis of myosin filaments from scallop striated muscle. *J. Mol. Biol.* 165:303-320.
- Vibert, P., and R. Craig. 1985. Structural changes that occur in scallop myosin filaments upon activation. *J. Cell Biol.* 101:830-837.
- Watanabe, S. 1985. Phosphorylation of chicken gizzard myosin: myosin filament hypothesis of calcium regulation. *Adv. Biophys.* 19:1-20.
- Wray, J. S. 1982. Organization of myosin in invertebrate thick filaments. In *Basic Biology of Muscles: A Comparative Approach*. B. M. Twarog, R. J. C. Levine, and M. M. Dewey, editors. Raven Press, New York. 29-36.
- Wray, J. S., P. J. Vibert, and C. Cohen. 1974. Cross-bridge arrangements in *Limulus* muscle. *J. Mol. Biol.* 88:343-348.