Effects of Phosphorylation by Myosin Light Chain Kinase On the Structure of *Limulus* Thick Filaments

Rhea J. C. Levine, Peter D. Chantler, Robert W. Kensler, and John L. Woodhead

Department of Anatomy and Neurobiology, Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129

Abstract. The results discussed in the preceding paper (Levine, R. J. C., J. L. Woodhead, and H. A. King. 1991. *J. Cell Biol.* 113:563–572.) indicate that A-band shortening in *Limulus* muscle is a thick filament response to activation that occurs largely by fragmentation of filament ends. To assess the effect of biochemical changes directly associated with activation on the length and structure of thick filaments from *Limulus* telson muscle, a dually regulated tissue (Lehman, W., J. Kendrick-Jones, and A. G. Szent Gyorgyi. 1973. *Cold Spring Harbor Symp. Quant. Biol.* 37: 319–330.) we have examined the thick filament response to phosphorylation of myosin regulatory light chains. In agreement with the previous work of J. Sellers (1981. *J. Biol. Chem.* 256:9274–9278), *Limulus* myosin, incubated with partially purified chicken gizzard myosin light chain kinase (MLCK) and [γ^{32}P]-ATP, binds 2 mol phosphate/mole protein. On autoradiographs of SDS-PAGE, the label is restricted to the two regulatory light chains, LC_1 and LC_2. Incubation of long ($\geq$4.0 μm) thick filaments, separated from *Limulus* telson muscle under relaxing conditions, with either intact MLCK in the presence of Ca^{2+} and calmodulin, or Ca^{2+}-independent MLCK obtained by brief chymotryptic digestion (Walsh, M. P., R. Dabrowska, S. Hinkins, and D. J. Hartshorne. 1982. *Biochemistry.* 21:1919–1925), causes significant changes in their structure. These include: disordering of the helical surface arrangement of myosin heads as they move away from the filament backbone; the presence of distal bends and breaks, with loss of some surface myosin molecules, in each polar filament half; and the production of shorter filaments and end-fragments. The latter structures are similar to those produced by Ca^{2+}-activation of skinned fibers (Levine, R. J. C., J. L. Woodhead, and H. A. King. *J. Cell Biol.* 113:563–572). Rinsing experimental filament preparations with relaxing solution before staining restores some degree of order of the helical surface array, but not filament length. We propose that outward movement of myosin heads and thick filament shortening in *Limulus* muscle are responses to activation that are dependent on phosphorylation of regulatory myosin light chains. Filament shortening may be due, in large part, to breakage at the filament ends.

We have previously reported that thick filaments separated from intact *Limulus* telson muscle, maintained in a relaxed state, have an average length >4 μm, whereas those obtained from similarly intact muscle, stimulated to contract isotonically either by exposure to high K^+ or electrically, have a mean length of 3 μm. This decrease in thick filament length is not the result of a rearrangement of component myosin and paramyosin molecules, since both long and shortened filaments have (*a*) identical helical surface arrays of myosin heads: threefold screw symmetry and fourfold rotational symmetry, and (*b*) identical diameters: the centers of mass of the myosin heads in both cases lie at a radius of 15 nm from the center of the filament shaft (Levine and Kensler, 1985). These results led us to suggest that *Limulus* thick filaments shorten by disaggregation of myosin and paramyosin from their ends.

More recently, we examined the effect of Ca^{2+} activation of detergent-skinned fiber bundles on A-band and thick filament structure. We found that both A-bands and thick filaments were shorter in activated bundles, maintained stretched beyond overlap of thick and thin filaments, then in skinned, unstimulated fibers and that short thick filaments were isolated from activated bundles that were allowed to shorten freely, but not from controls. Moreover, we observed the presence of numerous end fragments of thick filaments on grids of filaments separated from Ca^{2+}-activated fiber bundles and populating the I-bands in sections of activated bundles maintained at nonoverlap (Levine et al., 1991). Based on these results, we now hypothesize that thick filament shortening in *Limulus* muscle may be largely the result of end

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Dr. Kensler's current address is Division of Cell Biology and Biophysics, University of Missouri, Kansas City, MO 64110.

fragmentation, rather than just the disaggregation of individual component molecules.

Limulus muscle is dually regulated (Lehman and Szent-Gyorgyi, 1975): Ca^{2+} activates the contractile apparatus both by binding to troponin subunits on the thin filaments and by effecting a calmodulin-mediated phosphorylation of myosin regulatory light chains (MRLCs)[1] by endogenous myosin light chain kinase (MLCK) (Sellers, 1981). Two phosphorylatable LCs (LC_1 and LC_2) are present in *Limulus* myosin. A third LC (LC_3) is not phosphorylatable (Sellers, 1981). The distribution of the two RLCs is not known: they may be segregated between the two types of fibers present in the muscle (Levine et al., 1989), or localized preferentially in different parts of the thick filament, as occurs with myosin heavy chains in nematode body wall muscle (Miller et al., 1983). It is also possible that myosin molecules may be a heterodimers, having both RLCs. Our observations suggest that *Limulus* thick filaments are directly affected (to shorten) by activation. Since Craig et al. (1987) have shown that phosphorylation of MLCs has a disordering effect on the cross-bridge array of thick filaments separated from tarantula (a chelicerate arthropod related to *Limulus*) leg muscle, it is important to assess the effect of such phosphorylation in the *Limulus* system.

Here we report the results of an examination of the structural response of *Limulus* thick filaments to phosphorylation of MRLCs by intact chicken gizzard MLCK, in the presence of Ca^{2+} and calmodulin, and after brief chymotryptic digestion of the enzyme, to produce the Ca^{2+}-independent form (Walsh et al., 1982).

Materials and Methods

Protein Preparations

Limulus Myosin. Myosin was isolated from *Limulus* muscle by several cycles of extraction (in cold 0.6 M KCl, pH 7.8 in sodium bicarbonate)/reprecipitation (with 8–10 vol of ion-free water). 0.4 mM sodium azide (NaAz) and 0.1 mM PMSF were present in all media. All steps were carried out in the cold. Precipitated protein was dispersed in 0.6 M KCl by Potter homogenization. The precipitate from the last extraction cycle was rapidly homogenized in 0.6 M KCl, 5 mM ATP, 5 mM sodium phosphate buffer, pH 7.0 and centrifuged for 30 min at 45,000 *g* at 4°C, to remove actin. The supernatant was brought to 10 mM in ATP and 10 mM in $MgCl_2$ and myosin was separated by ammonium sulfate fractionation (the precipitate obtained between 42 and 65% saturation). Precipitated myosin, resuspended in a small volume of 0.6 M KCl containing: 2 mM $MgCl_2$, 0.5 mM EGTA and buffered with 5 mM Tris maleate (Trizma), pH 7.0, was dialyzed against the same buffer for 6 h, with three changes of dialysand. The protein concentration was determined spectrophotometrically at 280 nm by the extinction coefficient of myosin: an absorbance of 5.53 equal to 10 mg/ml using a quartz cell with a 1-cm light path. Generally 15–20 mg myosin were obtained from 60 g of muscle. The purity of the preparations were checked by SDS-PAGE on 12.5% minigel slabs.

Chicken Gizzard MLCK. Partially purified MLCK was prepared from the gizzards of fresh Kosher-killed chickens according to Adelstein and Klee (1982), omitting the last calmodulin affinity column purification step. The Ca^{2+}-independent form of the enzyme was produced by brief digestion with α-chymotrypsin of the partially purified MLCK, in the presence of 1.8 μM calmodulin (donated by Dr. Benjamin Weiss, Department of Pharmacology, Medical College of Pennsylvania) and 0.1 mM $CaCl_2$, according to the procedure of Walsh et al. (1982).

1. *Abbreviations used in this paper*: MLCK, myosin light chain kinase; MRLC, myosin regulatory light chain.

Incorporation of ^{32}P by Limulus Myosin

Limulus myosin was incubated with the intact gizzard MLCK by a modification of Seller's method (1981). The protein was dialysed versus 0.12 M NaCl, 10 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.5, containing a protease inhibitor cocktail (Sellers, 1981), to prevent Ca^{2+}-dependent protease activity. Incubations were carried out in 1–1.25-ml vol in testtubes at room temperature. All tubes contained 1 mg myosin, 3 μg calmodulin (generously donated by Dr. Benjamin Weiss), 2.5 mM ATP as [γ^{32}P]ATP, and appropriate buffer, to volume. Experimental tubes contained 0.5 ml of crude gizzard MLCK, and the buffer was 0.3 mM in $CaCl_2$. Controls were: (*a*) everything as above, but Ca^{2+} was omitted from the buffer, which was made 0.5 mM in EGTA; (*b*) with the substitution of 0.5 mg BSA for MLCK; (*c*) the same as (*b*), but without Ca^{2+} and with EGTA, as in (*a*). The tubes were incubated with their contents stirred for 1 h. The reaction was stopped by precipitating the protein by addition of 3 ml of a 1:10 dilution of the appropriate buffer (plus or minus calcium; plus or minus EGTA), containing 2% sodium pyrophosphate (NaPP) to maintain LC phosphorylation. After 10 min centrifugation at 3,000 *g*, the supernatants were discarded and the precipitates were resuspended in 1–2 ml of the appropriate diluted buffer without NaPP, and this step was repeated twice more. Finally, the precipitates were resuspended in 1.1-ml vol of the appropriate diluted buffers (minus NaPP) and dispersed by rapid stirring on a Vortex mixer for several seconds. Two 0.5-ml aliquots were removed from each tube and each aliquot was placed in a separate scintillation vial containing 5 ml of Aquasol scintillation fluid and shaken until transparent. The remaining 0.1 ml was used for determination of protein concentrations.

Blank vials included one with Aquasol fluid alone and two that each contained 0.5 ml of myosin at 4 mg/ml in Aquasol. Three [γ^{32}P]ATP sample vials contained: 107, 11, and 2.14 μmol labeled ATP, respectively. Radioactive decay/vial per minute was recorded using a Beckman LS6800 counter (Beckman Instruments Co., Fullerton, CA). All of the vials from each experiment were counted at least twice.

For each run, the counts per minute from the two tubes containing the aliquots from each protocol were averaged, as were those from the myosin blanks. The averaged background was subtracted from each averaged count. The radioactivity of 1 nmol of ATP for each run was obtained from the counts of the ATP samples. The experimental and control counts were then normalized on the basis of 1 nmol of myosin, using the protein concentrations determined for each incubation. The normalized results for identical incubations from five different runs were then averaged with each other. One experimental and control set of incubations was run on SDS-12% PAGE for autoradiography.

Isolation and Incubation of Thick Filaments

Thick filaments were separated from relaxed *Limulus* telson muscle fiber bundles as previously described (Kensler and Levine, 1982). Samples from each preparation were adsorbed onto medium-thickness carbon films on 300-mesh copper grids, negatively stained with 1% aqueous uranyl acetate, and examined in the electron microscope for appearance of the filaments. Preparations showing filaments with good helical order of surface myosin heads were used for experimental procedures.

All incubation media contained 0.12 M NaCl, 5 mM $MgCl_2$, 40 mM Tris-HCl, pH 7.0, 2.5 mM ATP and either full-strength, 1:10 or 1:100 dilutions of MLCK. When the intact kinase was used, the protease inhibitor cocktail (Sellers, 1981) was present in the buffer. Experimental incubations using intact MLCK included 2 mM $CaCl_2$ and 5 μM calmodulin. Controls for these incubations included omission of calcium from the medium and substitution of BSA for MLCK, with and without calcium. In two experiments, both calcium and calmodulin were omitted and similar dilutions of Ca^{2+}-independent MLCK were substituted for the intact enzyme. Controls for these experiments included substitution of BSA for MLCK or just the absence of the enzyme. Incubations were performed either (*a*) by inverting grids containing filaments adsorbed onto a carbon film, while still in a thin film of relaxing buffer, onto droplets of either experimental or control media (on parafilm) for 1–10 min (for incubations >2 min, the grids were moved onto the surface of three successive droplets of medium at approximately equal time intervals), or (*b*) by washing the filaments (adsorbed onto a carbon film on EM grids) with a stream of 20 droplets of incubation medium. In this protocol, the last droplet was allowed to remain on the surface of the grid for 1–3 min. After either incubation procedure, the grids were rinsed with the buffer used in the incubation medium, without ATP, but with 2% NaPP present, to maintain the phosphorylated state of the LCs, and negatively-stained with 1% uranyl acetate. In some cases, after experimen-

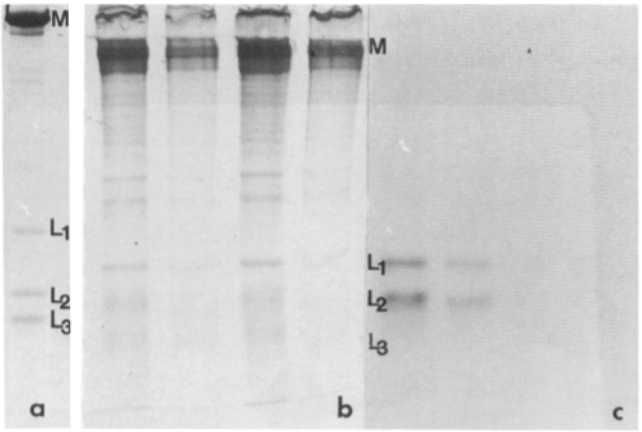


Figure 1. SDS-PAGE of isolated *Limulus* myosin preparations and an autoradiogram of an experimental/control [γ^{32}P]ATP incorporation set. *M*, myosin heavy chains; L_1,L_2, and L_3, myosin light chains, respectively. (*a*) SDS-12.5% polyacrylamide gel loaded with 15 μg protein. (*b*) SDS-12.5% polyacrylamide gel. Myosin in the two left lanes was incubated with MLCK, [γ^{32}P]ATP, calmodulin, and Ca^{2+}. Myosin in the two right lanes was incubated with MLCK, [γ^{32}P]ATP, calmodulin, but no Ca^{2+}. Myosin loadings were 15 μg and 7.5 μg myosin in each case. (*c*) Autoradiogram of gel shown in *b*. Note ^{32}P incorporation only into the myosin LC_1 and LC_2 bands, only when Ca^{2+} was present (*left lanes*).

tal incubations and rinsing with buffer, the grids were further rinsed with relaxing buffer containing 2.5 mM ATP (Kensler and Levine, 1982) before staining. The negatively-stained grids were examined and photographed at magnifications of 19,000 and 48,000 on a JEOL 100CX electron microscope operating at an accelerating voltage of 80 kV, with a 30-μm objective aperture in place and an anticontamination device in use. Magnifications were calibrated using a commercial grid with 2,156 lines/in.

Image Analysis

When electron microscopic examination indicated that the surface cross-bridge array was sufficiently ordered, optical diffraction patterns were obtained from the EM negatives, as previously described (Kensler and Levine, 1982).

Results

SDS-PAGE of Isolated Myosin

Isolated *Limulus* myosin, run on 12.5% SDS-PAGE, was relatively free of contaminating proteins (Fig. 1), although the degree of purity varied somewhat among different preparations. The MLCs are visible as distinct, low molecular weight bands (Fig. 1 *a*). Autoradiograms of SDS-PAGE of myosin incubated either with complete reaction medium or medium lacking just Ca^{2+}, showed that radioactive phosphate was confined to just the LC_1 and LC_2 bands, and only under experimental conditions (Fig. 1, *b* and *c*).

Incorporation of ^{32}P into Limulus Myosin

Fig. 2 shows the molar incorporation of labeled phosphate into *Limulus* myosin under experimental and four different sets of control conditions. The results of five different experiments are averaged. Myosin incubated with the complete experimental medium incorporated between 1.7 and 2.2 (mean 2.03) mol ^{32}P/mol protein, which is expected for *Limulus* myosin with two phosphorylatable light chains. Myosin alone, incubated with radioactive ATP, in the absence of MLCK, Ca^{2+}, and calmodulin incorporated almost no label at all ($\leq$0.03 mol ^{32}P/mol myosin). A similar result ($\leq$0.1 mol ^{32}P/mol myosin) was obtained with myosin incubated with BSA, [γ^{32}P]ATP, and calmodulin. Myosin incubated with gizzard enzyme plus calmodulin, but no Ca^{2+}, was slightly more radioactive ($\leq$0.3 mol ^{32}P/mol myosin). Among the controls, only myosin incubated with complete medium, but with BSA substituted for MLCK incorporated ^{32}P to any appreciable extent, and that was less than one-third of the experimental value ($\sim$0.62 mol ^{32}P/mol protein). It is possible that some endogenous MLCK present in the myosin preparation was active in this case. Together with the results of autoradiography, above, our findings indicate, that as first described by Sellers (1981), *Limulus* myosin contains two phosphorylatable light chains and that both are responsive to MLCK.

Effect of MRLC Phosphorylation on Relaxed Limulus Thick Filaments

Both native relaxed thick filaments, separated from *Limulus* fiber bundles in relaxing solution, and those that were incubated with intact MLCK without Ca^{2+} are long, averaging 4.22 μm (Fig. 3, *a* and *c*) and have an ordered helical array of surface myosin heads (Fig. 3, *b* and *d*). The preservation of order is slightly less obvious when filaments had been incubated in the control media. This appears to be due to a decrease in the quality of the staining, which is extremely sensitive to the addition of protein (MLCK) onto the surface of the grid. Nevertheless, diffraction patterns obtained from electron micrograph negatives of these filaments and those that were not incubated in any medium are similar and layer lines out to the sixth are present (Fig. 3, *e* and *f*). Thus, the incubation step, itself, does not affect thick filament structure, appreciably.

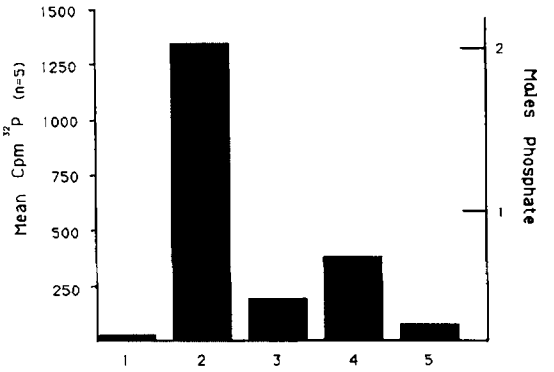


Figure 2. Histogram showing the incorporation of ^{32}P from [γ^{32}P]-ATP by isolated *Limulus* myosin under different conditions of incubation. Bar heights are the means from five different runs. Bar *1*, myosin alone, no MLCK; bar *2*, myosin, MLCK, Ca^{2+}, and calmodulin; bar *3*, myosin, MLCK and calmodulin, no Ca^{2+}; bar *4*, myosin, BSA, Ca^{2+}, and calmodulin; bar *5*, myosin, BSA and calmodulin, no Ca^{2+}. Counts per minute are displayed on the left *y*-axis; moles ^{32}P incorporated on the right *y*-axis (calculated from counts of [γ^{32}P]ATP). In the presence of MLCK, Ca^{2+}, and calmodulin, 1 mol of *Limulus* myosin incorporates 2 mol of phosphate.

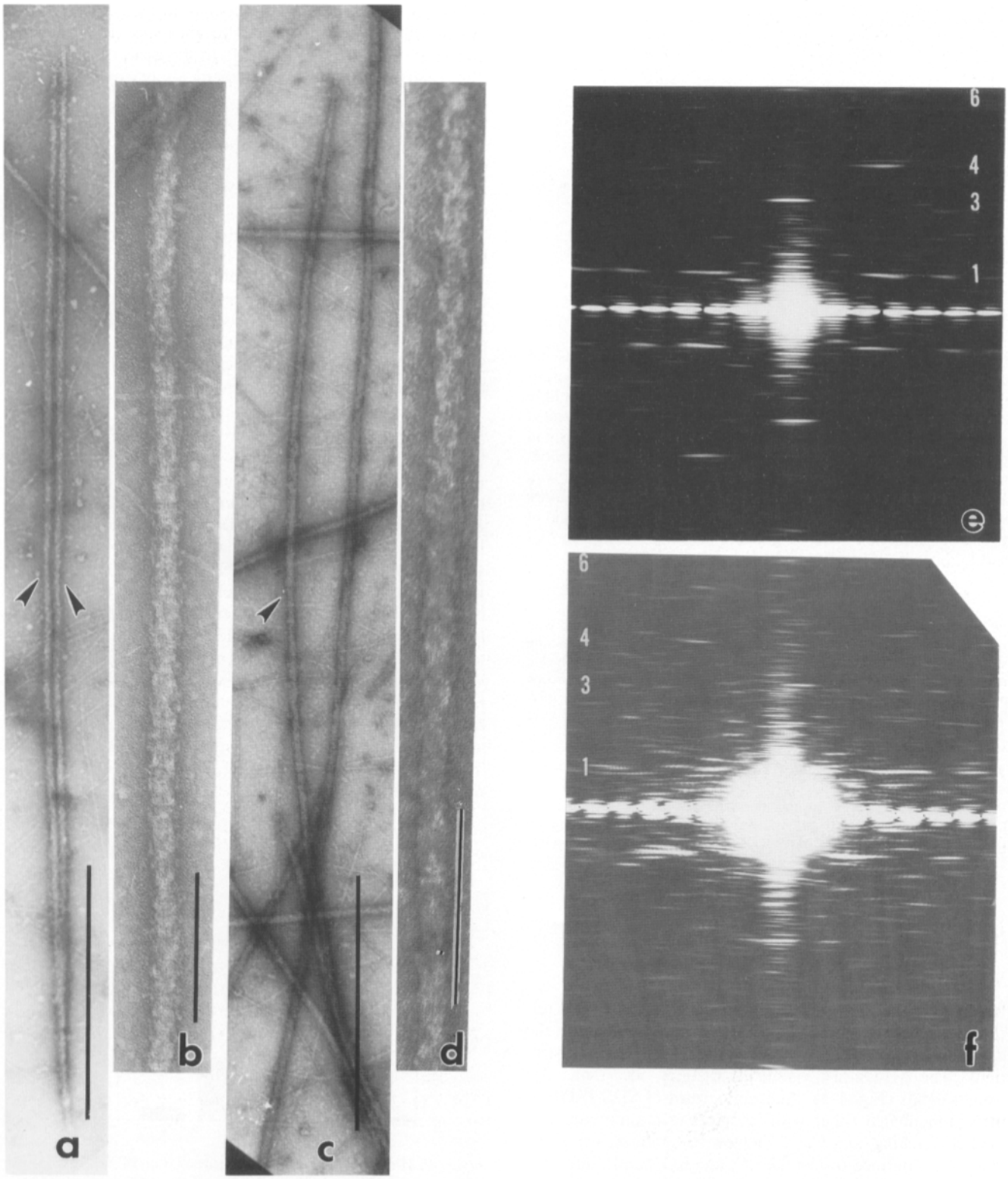
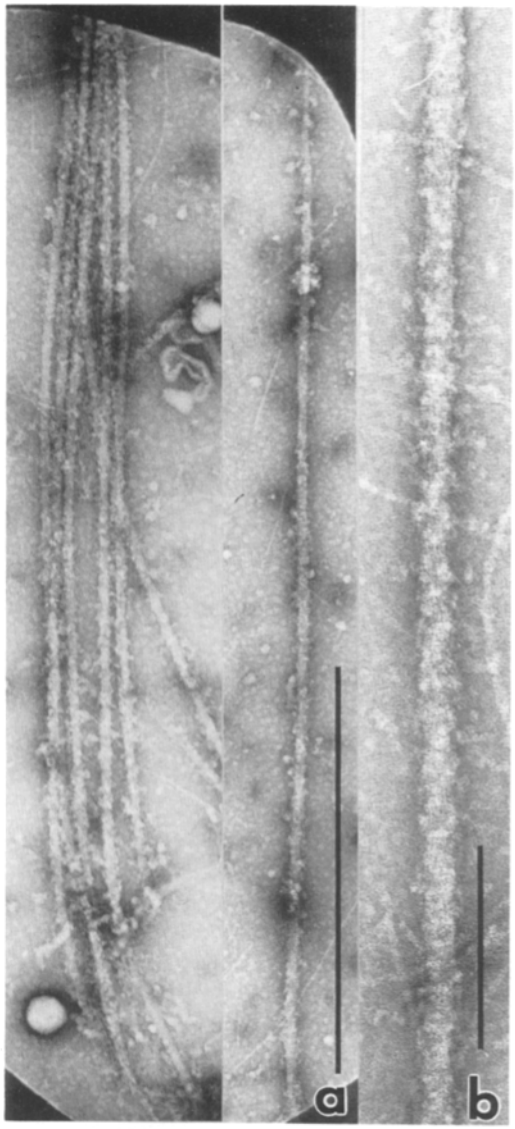


Figure 3. Electron micrographs and optical transforms of negatively stained, control, thick filaments, separated from unstimulated *Limulus* telson levators. Arrowheads indicate bare zones. Filaments in *a* and *b* were washed with relaxing solution before staining. (*a*) Low magnification electron micrograph. Note long filaments, with good helical surface order. (*b*) High-magnification electron micrograph. A portion of a thick filament is shown. The bare zone is down. Note the good helical order of the surface myosin array. Filaments in *c* and *d* were incubated in control medium (intact MLCK and calmodulin, but no Ca^{2+}). (*c*) Low-magnification electron micrograph showing long filaments. Some degree of surface order is still present, but the staining is not as good as when no additional protein is present. (*d*) High magnification electron micrograph of a portion of a similarly-incubated thick filament. The bare zone is down. Some surface order is preserved. (*e*) Optical transform from a filament like those in *a*. The relaxed myosin helical array is present, although the intensity change of reflections across the meridian indicates one-sided staining. Layer lines *1*, *3*, *4*, and *6* are visible. (*f*) Optical transform from the filament in *b*. Layer lines *1*, *3*, *4*, and *6* (numbered on the transform) are still present, but there is a great deal of background noise. Again, this may be due to additional protein (MLCK) present on the filaments. Bars: (*a* and *c*) 1 μm; (*b* and *d*) 0.2 μm.



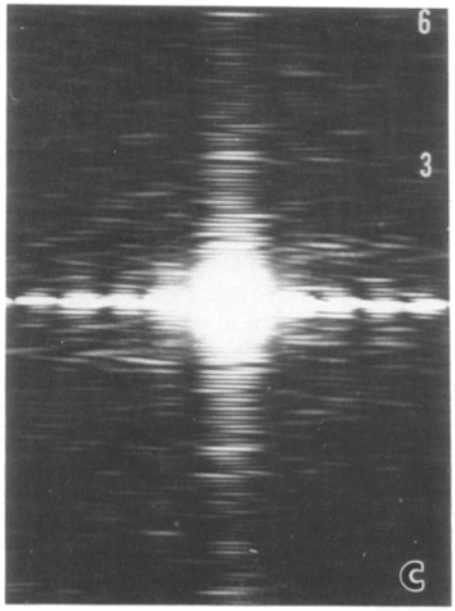


Figure 4. Electron micrographs and an optical transform of negatively stained *Limulus* thick filaments that were incubated in experimental media. (*a*) Low magnification electron micrographs. Filaments in left image were incubated with intact MLCK, calmodulin, and Ca^{2+}, in the presence of protease inhibitors. Filament in right image was incubated with Ca^{2+}-independent MLCK. Note the lack of surface order and general disarray in both. (*b*) High-magnification electron micrograph of a portion of a filament incubated with Ca^{2+}-independent kinase. The helical repeat of the surface lattice is disrupted. (*c*) Optical transform from the right-hand filament in *a*. The first meridional reflection on the third layer line (*11*; numbered *3*) is clear and there is a faint meridional reflection on the sixth layer line (*6*). The helical information is lost. Bars: (*a*) 1 μm; (*b*) 0.2 μm.

The appearance of thick filaments incubated either with intact MLCK in the presence of Ca^{2+} and calmodulin or with Ca^{2+}-independent MLCK, obtained by chymotryptic digestion, is vastly different from that of the controls described above (Figs. 4, 5, and 6). One difference, not related to the overall results, is that there is very poor staining of grids incubated with full-strength enzyme. By far, the best staining of experimental grids is seen when the 1:100 dilutions were used. Further, the diluted enzyme produces structural effects identical to those seen with full-strength MLCK. There are three major structural effects of MLC phosphorylation on filament structure. First, there is loss of helical order. Myosin molecules are seen to extend, in random fashion, for varying distances away from the filament shaft (Fig. 4 *a*). Fourier transforms of such filaments show a nearly complete loss of off-meridional layer lines (Fig. 4 *b*). A second, consistent, structural effect of MLCK activity is the presence of sharp bends or breaks in the distal region of one or both polar halves of many filaments (Fig. 5, *a* and *b*). Regions of the filament shafts near these bends occasionally appear smooth, indicating the loss of myosin molecules. Broken-off ends appear as fragments (Fig. 5 *c*) and these may occasionally be found associated with the thick filaments from which they derive. Third, most of the thick filaments on experimental grids are short: their average length is 3.02 μm (Fig. 6). The average length of end fragments is 0.69 μm. Thus, two end fragments plus one short thick filament have a total length equal to that of a control, long thick filament.

Without further exposure to relaxing solution, all the short filaments on experimental grids are highly disordered (Fig. 6, *a* and *b*). Rinsing of experimental grids with relaxing solution, prior to negative staining, restores some degree of surface order. Myosin molecules no longer extend away from the filament shaft (Fig. 6, *c* and *d*), and there is some restoration of the relaxed diffraction pattern (Fig. 6 *e*). There is however, no restoration of thick filament length: after a terminal rinse in relaxing solution, thick filaments that were exposed to experimental media remained at an average length of 3.13 μm (Table I).

Discussion

With this study of the effects of MLC phosphorylation, our examination of the effects of stimulation and activation on the structure of thick filaments, separated in the relaxed state from *Limulus* telson muscle, is brought to the level of molecular events.

The results described here demonstrate that our preparations of *Limulus* myosin are most likely unphosphorylated, since they are capable of incorporating ~2 mol ^{32}P/mol protein under appropriate conditions. We also show that our

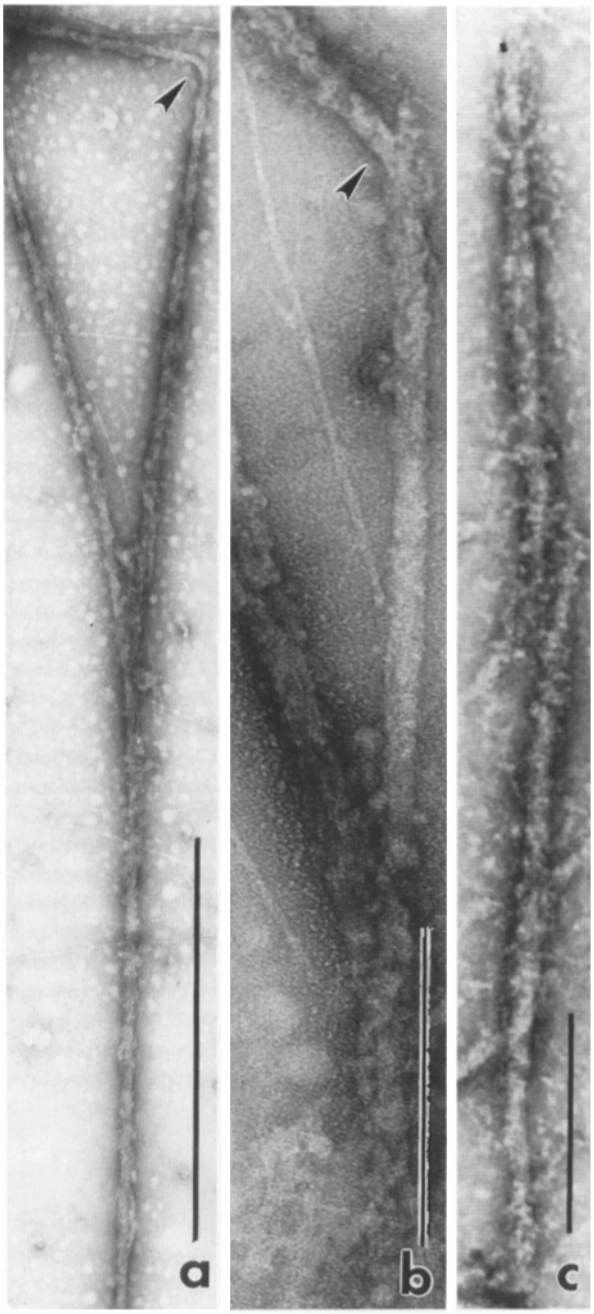


Figure 5. Electron micrographs of negatively stained *Limulus* thick filaments incubated in experimental media. (*a*) Low-magnification micrograph showing a distal bend in filament (*arrowhead*), together with extreme disorder of the surface myosin array. This filament was incubated with Ca^{2+}-independent kinase. (*b*) High-magnification micrographs of a bent regions in a filament that was incubated with Ca^{2+}-independent kinase. Note the "unraveling" of the surface array of myosin heads. (*c*) Two filament fragments from a preparation incubated with Ca^{2+}-independent kinase. Myosin is present both on the surfaces of the fragments and on the carbon support film. Bars: (*a*) 1 μm; (*b* and *c*) 0.2 μm.

crude preparation of chicken gizzard MLCK, whether intact (in the presence of Ca^{2+} and calmodulin) or chymotryptically cleaved (Ca^{2+} independent), is effective in phosphorylating the RMLCs of this arthropod myosin. We further confirm Sellers's (1981) results, demonstrating that both LC_1 and LC_2 of *Limulus* myosin are phosphorylatable. Thus, our results differ from those of Kerrick and Bolles (1981), who reported that only LC_1 of *Limulus* myosin was phosphorylated during Ca^{2+} activation of skinned fibers, in the absence of added MLCK.

Since *Limulus*-striated muscle is dually regulated, with both thick and thin filaments participating in switching contractile function "on" (Lehman and Szent-Gyorgyi, 1975) it is not unexpected for the helical array of surface myosin heads on *Limulus* thick filaments to become disordered, reaching out from the filament backbone, as a result of MLC phosphorylation. A number of studies (Bennett et al., 1984; Szentkiralyi, 1984; Winkelmann et al., 1984) have located the light chains at the head–rod junction of scallop striated muscle myosin, and it is likely that this is a widely occurring condition. By increasing the number of negative charges at head–rod junctions, RLC phosphorylation may induce outward movement of the myosin heads toward the thin filaments. Similar disordering effects of such activation-related phenomena as MLC phosphorylation and Ca^{2+} binding, on the surface array of myosin heads have been observed on thick filaments from tarantula leg muscle (Craig et al., 1987) and both native (Vibert and Craig, 1985) and synthetic (Frado and Craig, 1989) thick filaments from scallop striated adductors respectively. It is not known if the spider muscle is dually regulated like *Limulus* muscle, but the scallop tissue is a myosin-regulated system, depending only on Ca^{2+} binding to this protein for activation (Kendrick-Jones et al., 1970; Lehman and Szent-Gyorgyi, 1975; Chantler and Szent-Gyorgyi, 1980). Other environmental conditions can also induce disorder of the myosin surface lattice of thick filaments. We have previously demonstrated (Levine et al., 1986), in agreement with the earlier x-ray studies of Wray et al., (1974), that the ordered array of surface cross-bridges on *Limulus* thick filaments if sensitive to changes in ionic strength. Myosin heads also move away from the *Limulus* filament shaft with: increases in pH and ionic strength, depletion of ATP and/or Mg^{2+}, and in the presence of ethylene glycol. Most of these perturbations are readily reversible in relaxing solution (Levine et al., 1986). Scallop thick filaments exhibit a similar, also reversible, response to ATP depletion (Vibert and Craig, 1985).

Unique to *Limulus* thick filaments, however, is their tendency to undergo end-fragmentation and loss of some myosin molecules under phosphorylation conditions. We observe this effect with both intact MLCK (in the presence of Ca^{2+} and calmodulin) and Ca^{2+}-independent enzyme, but not in controls with BSA substituted for MLCK. Since protease inhibitors were present when the intact MLCK was used, and since Ca^{2+} was omitted from the media in preparations using the chymotryptically cleaved enzyme, it is unlikely that filament breakage is the result of protease activity. Furthermore, gels of myosin that had been phosphorylated under similar conditions show no differences in banding patterns from those of controls: low molecular weight degradation products that might result from protease activity are absent from experimental lanes.

The end-fragmentation, and resultant short thick filaments produced here by experimental incubations of relaxed (long) filament preparations, are effects similar to those seen after Ca^{2+} activation of skinned fiber preparations (Levine et al., 1991). Short thick filaments are also recovered from intact

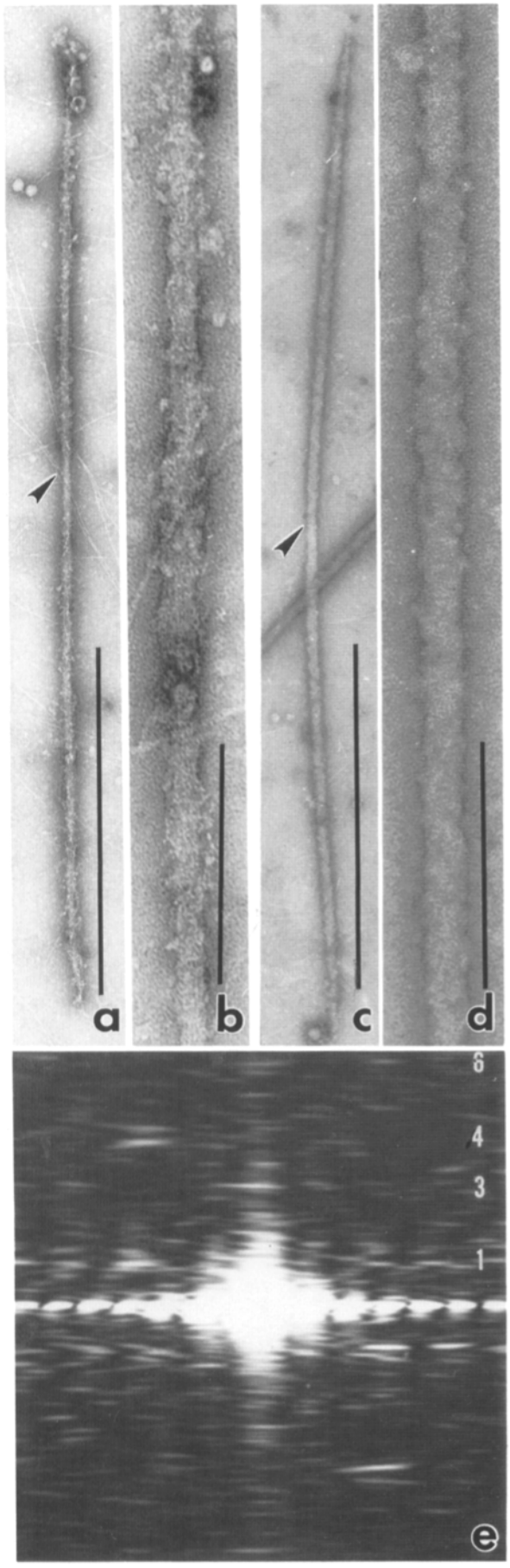
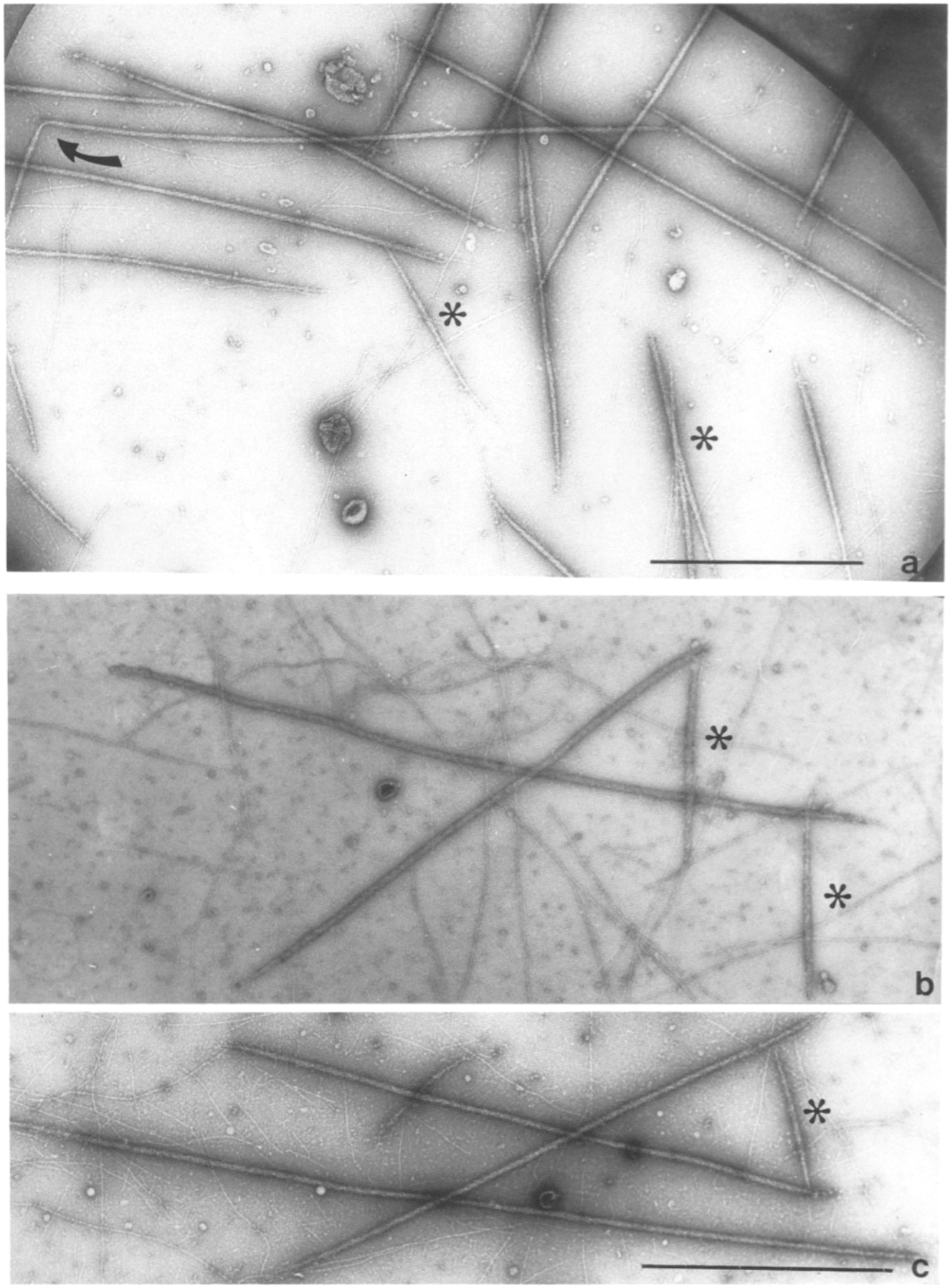


Table I. Response of Thick Filaments to Activation

Treatment	Mean thick filament length	Surface array
	μm/n	
Unstimulated intact fibers, filaments isolated in and washed with relaxing solution	4.26(392)	Ordered*
Stimulated intact fibers, filaments isolated in and washed with relaxing solution	3.14(350)	Ordered*
Skinned fibers, filaments isolated in and washed with relaxing solution	4.11(218)	Ordered
Ca^{2+}-activated skinned fibers, filaments isolated in and washed with relaxing solution	3.25(598)	Ordered
Unstimulated intact fibers, filaments isolated in relaxing solution, then incubated with MLCK and calmodulin (no Ca^{2+}) and rinsed with buffer	4.22(89)	Ordered
Unstimulated intact fibers, filaments isolated in relaxing solution, then incubated with MLCK, Ca^{2+}, and calmodulin and rinsed with buffer	3.02(187)	Disordered
Unstimulated intact fibers, filaments isolated in relaxing solution, then incubated with MLCK, Ca^{2+}, and calmodulin and rinsed with relaxing solution	3.13(77)	± Ordered

* Levine and Kensler (1985). Levine et al., 1991, 563-572.

Figure 6. Electron micrographs and an optical transform of negatively stained short *Limulus* thick filaments from experimental preparations. Arrowheads indicate bare zones. (*a*) Low-power electron micrographs of a short thick filament that was incubated with Ca^{2+}-independent kinase. Note the surface disorder and short length. (*b*) High-power electron micrograph of a portion of a filament incubated with intact MLCK, Ca^{2+}, and calmodulin, in the presence of protease inhibitors. The bare zone is down. Surface disorder is apparent. (*c*) Low-power electron micrograph of a short thick filament that was rinsed with relaxing solution after the experimental incubation with intact MLCK, Ca^{2+}, and calmodulin, in the presence of protease inhibitors, and prior to negative staining. The surface of this filament is smoother than that of the filament in *a*. (*d*) High-power electron micrograph of a portion of the filament seen in *c*. Again, note the smoother surface of the filament after a rinse in relaxing solution as compared with the filament in *b*. While there is not a pronounced return to a high degree of helical order, the myosin heads have moved back toward the filament backbones. (*e*) Optical transform of the filament shown in *c*. Again, the transform is one-sided, probably due to the stain, and there is considerable background present. Nevertheless, some reflections along layer lines *1*, *3*, *4*, and *6* (labeled) are present, indicating a degree of return of the helical surface array of myosin heads. Bars: (*a* and *c*) 1 μm; (*b*) 0.2 μm.


a
b
c

fiber bundles, stimulated to contract either by brief exposure to high K^+, or electrically (Levine and Kensler, 1985). Interestingly, reexamination of electron micrographs from that study also shows the presence of many filament end fragments on grids of stimulated, but not of unstimulated, preparations (Fig. 7). Thus, for *Limulus* thick filaments, the biochemical endpoint of activation is phosphorylation of the MRLCs. The structural endpoint appears to be the loosening of myosin–myosin and/or myosin-paramyosin interactions in the filament shaft, producing an outward movement of myosin heads and the release or bending of a portion of each end of the filament, effecting a decrease in filament length. The latter structural response to activation is observed whether the thick filaments are in situ, within the lattice of thick and thin filaments in the intact contractile apparatus, or are separated from the tissue and lying in a thin film of medium, supported on an EM grid.

The incomplete reordering of the surface lattice we observe after experimental preparations are rinsed with relaxing solution is primarily due to the movement of the myosin heads back toward the filaments' shafts. At this time we do not know if dephosphorylation of the MLCs has occurred or if this effect is solely the result of ATP binding to the S1 active sites.

The phenomenon of end fragmentation of *Limulus* thick filaments raises many obvious questions: How does phosphorylation of MRLCs cause filament breakage? Are core paramyosin molecules involved, and if so, how? Where do the release end-fragments go, in an intact fiber? Under what conditions do they reanneal to the rest of the filament? How does filament shortening relate to tension development and isotonic contraction, in vivo? What is the adaptive advantage of thick filament end fragmentation for the horseshoe crab? Do the muscles of any other species exhibit the same phenomenon?

So far, one can only speculate about the answers to most of these questions. There are no reports suggesting that the muscles of other species exhibit thick filament shortening of the same magnitude as those of *Limulus* (25–30%), although the book is not yet closed: in jellyfish fishing tentacle muscle thick filaments undergo a structural change between the extended and retracted state of the organ (Perkins et al., 1971). *Limulus* is one of the oldest extant arthropod species, appearing in its present form in the Mesozoic Era, and, among the arthropod group, has an unusual arrangement of fibers in the muscles responsible for telson (tailspine) movement and for flexion of the joint between the prosomal (front) and opisthosomal (rear) carapaces. The fibers run in a fairly straight course, for up to 7 or 8 cm (depending on the size of the animal), from origin to insertion. Since the dorsal segments are fused in *Limulus*, individual fibers and their component sarcomeres, particularly those of the telson levator muscles, undergo large changes in length between their fully shortened (telson held perpendicularly up with respect to the opisthosomal carapace) and fully extended (telson held down at ~60° to the opisthosomal carapace) states. For the telson to function as a lever to right the crab when it is upside down and also as a rudder in swimming, requires its full, >180°, range of movement in the vertical plane as well as equally large rotational movements (Eagles, 1973; Silvey, 1973). As we have earlier shown, the sarcomeres of large-diameter fibers (Levine et al., 1989) undergo at least a two-fold change in length from >9.0 to <4.0 μm, as do those of small diameter fibers (Levine et al., 1989), from >12.0 to <6.0 μm, as they shorten to produce full elevation of the telson from a fully depressed position. In each of these fiber types, the length of the "relaxed" thick filaments is longer than that of the fully shortened sarcomeres. *Limulus* telson muscle fibers have complete Z-discs (Dewey et al., 1973), rather than perforated ones like those of the scutal depressors of the giant barnacle, *Balanus nubilis* (Hoyle et al., 1965; Leyton and Ullrick, 1980). Therefore, unlike the situation in the barnacle muscle, *Limulus* thick filaments cannot pass through the Z-discs into adjacent sarcomeres, when the muscle is fully shortened. The ability of the end regions to be reversibly bent or dissociated from the thick filaments, however, may function to permit full fiber shortening and freedom of telson movement.

Since MRLC phosphorylation appears to be causally related to filament end fragmentation, it will be important to determine the distribution of the two phosphorylatable light chains along the thick filament. Possibly, the myosin along most of the length of the filament bears only one RLC, while both are present in the region affected by activation. Such a topological distribution of two myosins is found in the thick filaments of the nematode, *Caenorhabditis elegans* (Miller et al., 1983; Epstein et al., 1986). Conceivably, a double dose of negative charge, resulting from phosphorylation of two MRLCs/myosin, promotes increased loosening and/or dissociation of component molecules in a discrete portion of each thick filament arm. It is likely that this is a reversible process, since long thick filaments are obtained from unstimulated muscle, although the animal has previously used its telson (and presumably contracted its muscles) many times before we entered the scene. Nevertheless, the mechanism governing reformation of long thick filaments is not known. We have recently reported that titin-like structures appear to be attached to the distal one-thirds of *Limulus* thick filaments, and to filament end-fragments that become dislocated into the I-bands in super-stretched, activated fibers (Levine et al., 1991). Complete fragmentation, however, need not occur in vivo, in fibers operating over their physiological range of sarcomere lengths, where there is overlap of thick and thin filaments. The loosened molecules may allow the thick filament ends to fold back, while being retained within the A-bands by connections either to elastic, cytoskeletal elements similar to titin or, via cross-bridge formation, to surrounding thin filaments, during the full range

Figure 7. Electron micrographs of negatively stained *Limulus* thick filaments that were separated from telson levator bundles that were stimulated to contract isotonically by brief exposure to high K^+ (Levine and Kensler, 1985). (*a*) Low-magnification image of a field of filaments. Note the presence of a bend (*arrow*) in one filament and of filament fragments (*asterisks*). (*b* and *c*) Low-to-medium-magnification images of groups of filaments, showing sharp distal bends and/or breaks and the presence of filament end-fragments (*asterisks*). Bars: (*a* and *c*) 1 μm.

of isotonic contraction. the ends would then be in position to reanneal to the body of the thick filaments. A decrease in available sites for production of active tension, as would occur with either folding or end fragmentation, should be reflected by an unusual length-tension relationship in the fibers. We plan to examine this physiological parameter in skinned, single fibers. We also plan to determine the distribution of *Limulus* myosin light chains in sarcomeres and thick filaments, using monoclonal antibodies.

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