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X-ray diffraction study of the structural changes accompanying phosphorylation of tarantula muscle

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Summary

Electron microscopy of negatively stained isolated thick filaments of tarantula muscle has revealed that phosphorylation of myosin regulatory light chains is accompanied by a loss of the helical order of myosin heads. From equatorial X-ray diffraction patterns of tarantula muscles in the phosphorylated state we have detected a mass movement in the myosin filaments that supports this finding.

Introduction

Muscular contraction is controlled by specific regulatory proteins that switch the actin–myosin interaction ON or OFF in response to changes in Ca^{2+} concentration. Two basic regulatory systems have been described: the actin-linked regulatory system, which involves the regulatory proteins troponin and tropomyosin; and the myosin-linked system, which involves the regulatory light chains associated with the myosin heads. In the myosin-linked regulation system, two pathways have been detected: activation by binding of Ca^{2+} directly to the myosin molecules (Kendrick-Jones *et al.*, 1970; Szent-Györgyi *et al.*, 1973) and activation by phosphorylation of the regulatory light chains (P-LC) (Adelstein & Eisenberg, 1980; Kendrick-Jones & Scholey, 1981).

In structural terms the mechanism by which Ca^{2+} regulates contraction by phosphorylation of the regulatory light chains is not yet understood. This problem has been studied by electron microscopy of negatively stained isolated thick filaments from tarantula muscle, which are an appropriate system because they present regulation by phosphorylation of the regulatory light chains (Craig *et al.*, 1987) and the structure of the thick filaments is known at 5 nm resolution (Padrón *et al.*, 1984; Crowther *et al.*, 1985). In the relaxed state the filaments display an ordered helical arrangement of myosin heads, in which the heads from axially neighbouring myosin molecules appear to interact with each other (Crowther *et al.*, 1985); that structure is also supported by chemical crosslinking studies (Levine *et al.*, 1988). When the regulatory light chains are phosphorylated the helical myosin heads arrangement of the relaxed filaments is lost, and the myosin heads sometimes appear clumped or to project further from the filament backbone (Craig *et al.*, 1985, 1987). Similar results have been reported for *Limulus* thick filaments (Levine, 1986). To explain these changes it has been proposed (Crowther *et al.*, 1985) that in the relaxed state (light chains non-phosphorylated) the myosin heads are held down on the filament backbone by head-head or head-backbone interactions, inhibiting their interaction with actin. When the light chains are phosphorylated (Craig *et al.*, 1987), these interactions are loosened, leading to the observed disorder of the myosin heads and facilitating their interaction with actin. However, it is possible that the disordering shown by electron microscopy could be due to the negative staining procedure itself. In this paper we use an alternative method (low angle X-ray diffraction of whole muscles) to test the hypothesis, avoiding any preparative or staining artifact and giving instead information on intact muscle in near physiological conditions.

The changes in the helical order of the crossbridges caused by light chain phosphorylation observed by electron microscopy (Craig *et al.*, 1987) suggests a mass movement in the axial projection of the thick filaments which should be detectable in the equatorial section of the X-ray diffraction pattern. Sosa and colleagues (1986, 1988) have recently been able to obtain X-ray diffraction patterns of tarantula muscle in the relaxed and rigor state, thus opening the possibility of using this technique to study the structural changes associated with the phosphorylation. In this paper low-angle X-ray diffraction has been used to study the structural changes that occur in the thick

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filaments of whole muscle from tarantula induced by the phosphorylation of the myosin light chains. We have obtained the equatorial X-ray diffraction patterns in the relaxed and phosphorylated states, which show differences in the relative intensities of the first three equatorial reflections, (10), (11) and (20), between both states. The intensity difference indicates that in skinned tarantula striated muscles regulatory light chain phosphorylation produces an outward movement of mass from the thick filament backbones. These results have been reported previously in preliminary form (Sosa *et al.*, 1987; Panté *et al.*, 1988).

Materials and methods

Specimens

Tarantulas *Avicularia avicularia*, kindly classified by Dr Sylvia M. Lucas (Divisão de Biologia, Instituto Butantan, São Paulo, Brazil), were collected locally and kept in a terrarium till use.

Muscle specimen preparation

The tarantulas were kept cold at 4° C for 1 h. Their legs were then removed and dissected at room temperature in cold spider Ringer (190 mM NaCl, 2 mM KCl, 4 mM $MgCl_2$, 4 mM $CaCl_2$, 1 mM Na_2HPO_4, pH 7.8, 370 mOsm kg^{-1}; Shartau & Leidescher, 1983). The freshly dissected muscles from the femur (first proximal segment) were skinned by the procedure developed by Padrón and Huxley (1984) and adapted for tarantula muscles (Sosa, 1986; Sosa *et al.*, 1988). The muscles were transferred to vials containing 25 ml of ice-cold skinning solution, consisting of fresh relaxing solution (100 mM K acetate, 5 mM Mg acetate, 5 mM EGTA, 15 mM K phosphate pH 6.3, 5 mM NaN_3, 2% (w/v) polyvinylpyrrolidone PVP-40, 5 mM ATP), containing 0.5% (w/v) Triton X-100. The vials were continuously agitated at 60 rev min^{-1} at 4° C for 3 h. The muscles were then rinsed several times with fresh relaxing solution at pH 7.2, to remove any traces of the Triton X-100. Then they were mounted in the X-ray cell following the procedure of Sosa and colleagues (1988) with relaxed, rigor or phosphorylating solution (pH 7.2), to obtain the equatorial X-ray diffraction patterns in the different states. X-ray patterns were recorded at 4° C. The rigor solution was identical to the relaxing solution but without ATP. The phosphorylating solution was the same as the relaxing solution but with 25 μg ml^{-1} Ca^{2+}-insensitive myosin light chain kinase (MLCK) and 1 mM ATP-τ-S (Boehringer Co.) instead of 5 mM ATP to produce an irreversible thiophosphorylation of the light chains (Morgan *et al.*, 1976).

Low-angle X-ray diffraction

Skinned tarantula leg muscles (about 18 mm long, 3 × 1.5 mm section) were mounted in small volume (about 5 ml) X-ray cells with two thin Mylar™ windows allowing the X-rays to pass through (Sosa, 1986), the sarcomere length was adjusted to its rest length value (7 μm) by laser diffraction, using a He/Ne laser (wavelength $\Gamma = 0.6328$ μm). After taking the X-ray diffraction pattern of the relaxed state, rigor or phosphorylating solution was introduced by means of a syringe after careful removal of the previous solution. A resistive transducer (UFI) and a preamplifier (Analog Devices AD521KD) were used to monitor the mechanical tension continuously during each experiment, using a recorder and oscilloscope. All experiments were done at 4° C.

Small-angle equatorial diffraction patterns were obtained using a mirror-monochromator camera of the type described by Huxley and Brown (1967) (cf. Sosa, 1986; Sosa *et al.*, 1986). The CuK_α radiation (wavelength $\Gamma = 0.154$ nm) from a Siemens Kristalloflex 810 X-ray generator was selected and focused horizontally by a 7° asymmetrically cut quartz crystal monochromator placed 19.5 cm from the X-ray source. Horizontal and vertical slits were used to cut down secondary scattering from the optical system. A vacuum tube enclosing most of the X-ray path between the specimen and the film was used to minimize air scattering. Diffraction patterns were recorded on CEA Verken films. Specimen-to-film distance was 51.1 cm and exposure times 5–10 h.

The optical density of the film was measured with a densitometer (Joyce Loebl MK III). A background line was drawn on each pattern by hand, and the intensity of each reflection was estimated by weighting the area of paper below each reflection without including the background. Due to limitations in spatial resolution of the patterns, the areas below the (11) and (20) peaks overlap and in some cases both reflections fuse in a single peak. We separate the contribution of each reflection by tracing by hand a gaussian curve at the position corresponding to the (11) and (20) reflections; these positions were estimated from the spacing of the (10) reflection assuming a hexagonal lattice.

Phosphorylation of myosin light chain kinase (MLCK)

MLCK was prepared by the procedure of Adelstein and Klee (1981) as described previously (Kendrick-Jones *et al.*, 1983). Calcium-insensitive MLCK was prepared by tryptic digestion of MLCK in the presence of bound calmodulin (Adelstein *et al.*, 1981). MLCK (0.5 mg ml^{-1}) in 100 mM NaCl, 25 mM TRIS-HCl pH 7.5, 0.2 mM $CaCl_2$, 2 mM dithiothreitol (DTT) with excess calmodulin was digested with trypsin (Sigma T-8128, Sigma Chemical Co.) at 250:1 for 1 h on ice. Digestion was terminated with a 10-fold excess of trypsin inhibitor (Sigma T-9253, Sigma Chemical Co.). Calmodulin was prepared from bovine brain as described previously (Scholey *et al.*, 1981).

Phosphorylation of the muscle specimen was performed directly in the X-ray cell. After changing from relaxing solution to phosphorylating solution, the temperature was increased to 25° C for 30 min to permit phosphorylation of the light chains. The temperature was then returned to 4° C and the X-ray diffraction pattern was recorded.

The extent of phosphorylation of the light chains was determined by urea/glycerol polyacrylamide gel electrophoresis (Perrie & Perry, 1970). The muscles were prepared for gel electrophoresis by the following procedure (Panté, 1986): the muscles used in the X-ray experiments were cut into small segments and homogenized for 1 s. The suspension was treated with ice cold 3% (final concentration) TCA, and the precipitated protein centrifuged. The pellet was washed once with ice cold acetone, dissolved in 9 M urea, 80 mM TRIS-HCl, 500 mM Glycine, pH 8.6, 5 mM DTT, 0.01% Bromophenol Blue, and run on 10% acrylamide-40% glycerol, 20 mM TRIS-HCl 122 mM Glycine pH 8.6 gels. The protein bands on the gel were

visualized by a modification of the silver strain technique reported by Morrisey (1981).

Results

Muscle response to relaxing and rigor solutions

Skinned tarantula muscles, immersed in relaxing solution did not develop any tension. When the ATP in the solution was removed, however, the muscles started to develop rigor tension immediately as shown in Fig. 1. The rigor contracture tension was 10–15 mN and the time necessary to reach the plateau tension at 4° C was 20–60 min. When the rigor solution was changed to the relaxing solution, the muscles relaxed again (Fig. 1).

Equatorial X-ray diffraction patterns obtained from skinned tarantula muscles in relaxing solution show three equatorial reflections (Fig. 2a), which indexed as the (10), (11) and (20) of a hexagonal lattice with a spacing of 56 ± 1 nm. The ratio $d_{10}/d_{11} = 1.7$ for live muscle is similar to the one predicted for a hexagonal array ($\sqrt{3}$). Figure 2a–c show the equatorial patterns when relaxation–rigor–relaxation cycles were carried out on the same specimen. When the muscles go into rigor the (11) and (20) intensities increased and the (10) intensity decreased and this reversed when the muscle relaxed again. Figure 3 shows the averaged intensity ratios I_{10}/I_{11} and I_{10}/I_{20} obtained in each assayed condition. These changes have been interpreted in other muscles as a movement of the myosin heads toward the actin filaments (Huxley, 1968; Miller & Tregear, 1972; Wray *et al.*, 1974; Millman & Bennet, 1976; Wakabayashi & Namba, 1981; Padrón & Huxley, 1984; Yu *et al.*, 1985). The mechanical and structural responses of the muscles to relax and rigor solutions indicate that they were properly skinned. X-ray equatorial patterns were obtained also from live muscles in which it was verified that the specimens were twitching in response to electrical stimulation before and after the X-ray exposure. The X-ray

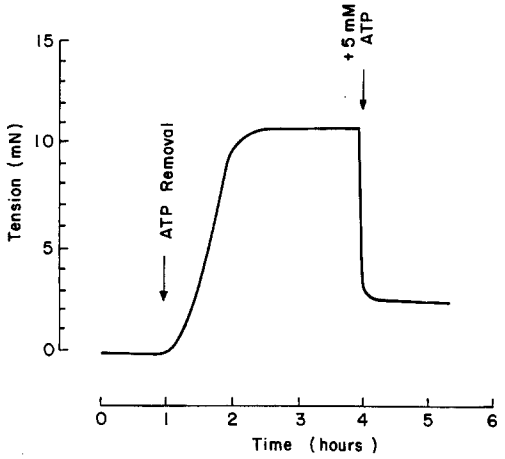

Fig. 1. Mechanical tension time-course during a relaxed-rigor-relaxed cycle for a skinned whole tarantula leg muscle. At sarcomere length 6.9 μm, under isometric conditions.

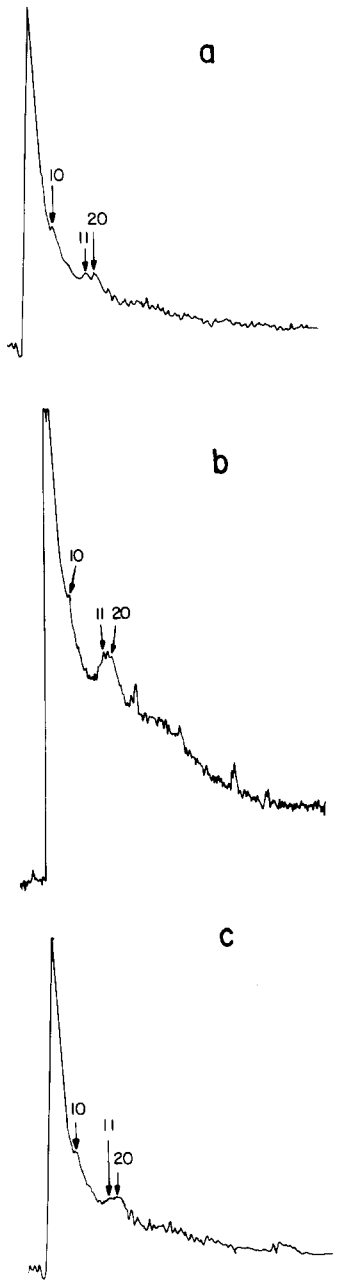

Fig. 2. Equatorial X-ray diffraction patterns, recorded from a skinned whole tarantula leg muscle: (a): in relaxing solution; (b) in rigor solution, showing the characteristic change in the I_{10}/I_{11} intensity ratio (i.e. $I_{11} > I_{10}$); and (c): after washing away the rigor solution with relaxing solution, showing a substantial recovery of the original I_{10}/I_{11} ratio. These changes in the equatorial X-ray diffraction pattern were correlated with mechanical changes as shown in Fig. 1. At sarcomere length 6.9 μm, and under isometric conditions, exposure time 10 h for each pattern.

pattern for the live relaxed muscle was similar to the X-ray pattern of demembranated relaxed muscle (Fig. 2a, cf. Sosa *et al.*, 1986, 1988). A similar intensity value for the (11) and (20) reflections, as reported in the present work, is consistent with a myofilament lattice with more than six thin filaments around each thick one (Millman & Bennet,

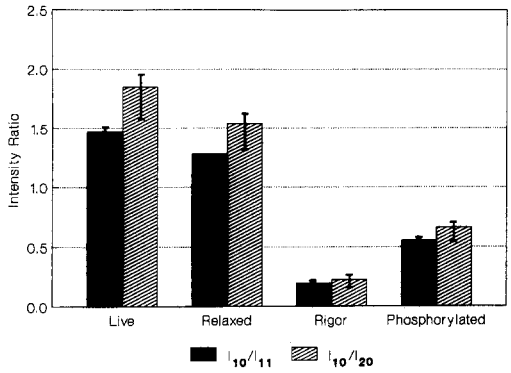

Fig. 3. Intensity ratios I_{10}/I_{11} and I_{10}/I_{20} for the different states assayed: intact live muscle; relaxed, rigor and phosphorylated demembranated muscle. The bars show the averaged value of six experiments ± SEM.

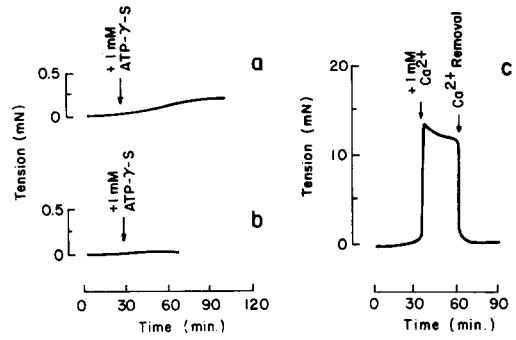

Fig. 4. Mechanical tension time-course generated during the phosphorylation of the light chains (phosphorylating solution), in the absence (a) and presence (b) of 5 mM ATP. (c) shows the mechanical tension time-course generated by a relaxed demembranated muscle when Ca^{2+} is added. At sarcomere lengths 7.0, 7.2 and 6.8 μm respectively for (a), (b) and (c), and under isometric conditions.

1976; Wakabayashi & Namba, 1981; Yagi & Matsubara, 1977). In fact, electron micrographs of cross-sections of the muscle used show a variable number of about ten (Sherman & Luff, 1971; Guerrero, 1990).

Phosphorylation experiments

We studied the mechanical and structural changes associated with the phosphorylation of myosin regulatory light chains of tarantula muscle. Light chain phosphorylation was performed in skinned muscle by diffusing into the muscle a phosphorylating solution (at 5 mM EGTA to maintain low Ca^{2+} levels) which contains a Ca^{2+}-insensitive MLCK. We used ATP-τ-S instead of ATP because thiophosphorylated proteins are not a favourable substrate for phosphatases (Sherry *et al.*, 1978). In this way, the possibility that the muscle endogenous phosphatases could reverse the phosphorylation was minimized. The mechanical tension and the equatorial X-ray patterns were simultaneously recorded in the same muscle, first in the relaxed state and then in the phosphorylated state. After each experiment, the state of phosphorylation of the light chains was verified by urea/glycerol gel electrophoresis.

Tarantula muscle in the phosphorylating solution developed some tension (Fig. 4a), but only about 2% of the full response in the presence of Ca^{2+} (Fig. 4c). This tension generation in the phosphorylated state was probably due to the low concentration of nucleotide (1 mM ATP-τ-S) since the same phosphorylation medium but with an additional 5 mM ATP does not generate tension (Fig. 4b).

Figure 5 shows a comparison between the equatorial X-ray diffraction patterns obtained from a relaxed muscle and a phosphorylated muscle. After phosphorylation the intensities of the reflections (11) and (20) increased, whereas the intensity of the reflection (10) decreased. These changes are in the same direction as when the muscle goes from the relaxed to the rigor state, but of less magnitude (Fig. 3). These changes are consistent with a movement of the myosin heads away from the thick filament backbone (see discussion). X-ray diffraction patterns were also recorded with a phosphorylating solution which contained 5 mM ATP in addition to the 1 mM ATP-τ-S. In this solution the changes in the equatorial X-ray pattern were the same as those shown in Fig. 5b (data not shown). Therefore the changes in the X-ray pattern are not due to the presence of some crossbridges in the rigor state, but are due to structural changes that occur during phosphorylation of myosin light chains. Owing to the long exposure time required to record the equatorial X-ray pattern on film, we have not attempted to reverse phosphorylation using a phosphatase.

The urea/glycerol gels of tarantula muscles demonstrated that the myosin light chains were phosphorylated after recording the X-ray pattern, as shown by the different mobility of the non-phosphorylated samples (lanes 1 and 3, Fig. 6) and phosphorylated sample (lane 2, Fig. 6). In some experiments [^{32}P]-τ-ATP was used to monitor that phosphorylation occurs (cf. Craig *et al.*, 1987).

Discussion

In this paper we report that phosphorylation of the regulatory light chains (26 kDa) P-LC of tarantula muscle produces changes in the equatorial X-ray diffraction pattern that are consistent with an outward movement of the myosin heads away from the thick filament backbone.

The structural changes associated with light chain phosphorylation were studied in skinned muscle. Both mechanical and X-ray measurements indicated that these muscle preparations were properly demembranated (cf. Padrón & Huxley, 1984, Sosa *et al.*, 1988). Mechanical records (Fig. 1) show that demembranated muscles remain relaxed when immersed in relaxing solution, but develop a rigor contracture when ATP is removed. The equatorial X-ray patterns of demembranated muscles are similar to

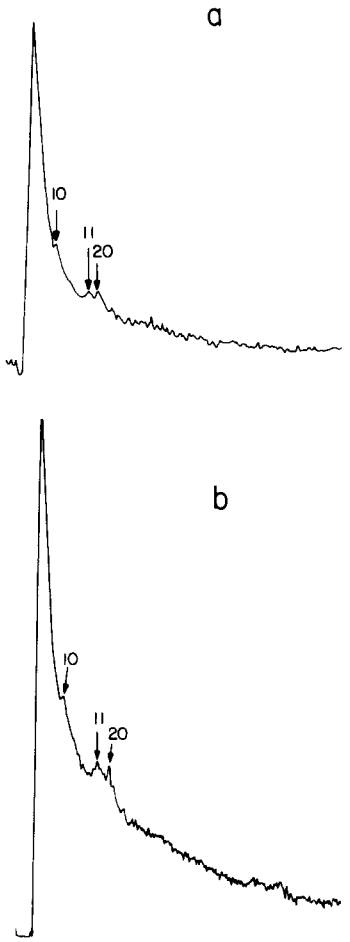

Fig. 5. A comparison of the equatorial X-ray diffraction patterns, recorded from two specimens of skinned whole tarantula leg muscles: (a): in relaxing solution; (b) in phosphorylating solution, after phosphorylation of the light chains showing a different intensity ratio I_{11}/I_{10} from the relaxed state. At sarcomere length 6.9 and 7.2 μm respectively, under isometric conditions, exposure time: 10 h each.

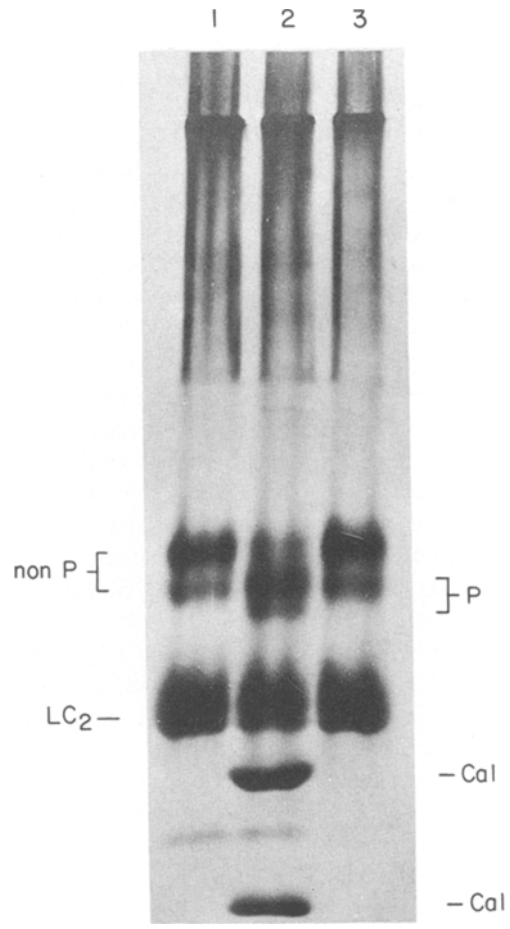

Fig. 6. Polyacrylamide gel electrophoresis (urea/glycerol of samples from relaxed (lanes 1 and 3) and phosphorylated (lane 2) muscle specimens (cf. Craig *et al.*, 1987, Fig. 2). non P denotes the positions of the phosphorylatable regulatory light chains LC_1 (26 kDa) in relaxing medium (presumed to be mainly non-phosphorylated), and P marks their new positions after incubation in phosphorylating solution with a Ca^{2+}-insensitive MLCK and ATP-τ-S (LCl phosphorylated). LC_2 denotes 'essential' light chains (19 kDa) and Cal denotes intact Calmodulin and a fragment.

those of the corresponding live muscle (cf. Sosa *et al.*, 1988), but with slightly smaller I_{10}/I_{11} and I_{10}/I_{20} ratios, due probably to some disordering of the filament lattice occurring during demembranation. When the solution bathing the muscle specimen was changed to the rigor condition, there was a clear change in the intensities of the equatorial X-ray diffraction pattern, the (11) and (20) reflection intensities increasing relative to the (10) (see Fig. 3). These changes are similar to the ones that occur in other muscles (Huxley, 1968; Miller & Tregear, 1972; Wray *et al.*, 1974; Millman & Bennet, 1976; Wakabayashi & Namba, 1981; Padrón & Huxley, 1984; Yu *et al.*, 1985) which have been interpreted as a movement of the myosin heads toward the actin filaments.

In the phosphorylated state we observed a similar change in the intensities of the (10), (11) and (20) reflections (Fig. 3) but of less magnitude. These changes occur without a concomitant rigor contracture (Fig. 4a). These X-ray results are consistent with a movement of the myosin heads away from the thick filament backbone but are different than with the rigor state (where there is cross-bridge formation and a rigor contracture) they are free to move. Preliminary modelling based on the computed map of 5 nm resolution three-dimensional reconstruction of tarantula muscle thick filament reported by Crowther and colleagues (1985) indicates that this movement is about 6 nm (Pante *et al.*, 1986).

The phosphorylation induced changes in the X-ray diffraction patterns are associated with phosphorylation of the myosin P-LC, as shown by the different mobilities of the P-LC in the urea-glycerol gel electrophoresis (Fig. 6, cf. Craig *et al.*, 1987).

Our X-ray diffraction results are consistent with the hypothesis proposed by Crowther and colleagues (1985) and Craig and colleagues (1987) that in the relaxed state

the myosin heads interact with the backbone or each other, maintaining a tightly packed helical array; whereas when the P-LC are phosphorylated, the heads are released from the thick filament surface ready to interact with the actin. Craig and colleagues (1987) showed that phosphorylation of the regulatory light chains produces disorder of the myosin heads but did not report any consistent outward movement of myosin heads. Alamo and Crowther (personal communication) have carried out a three-dimensional reconstruction of electron micrographs of negatively stained thick filaments of tarantula muscle in the phosphorylated state supporting disorder of the heads away from the relaxed position and finding no new ordered state. Our results on intact muscle in aqueous solution support the hypothesis derived from negative staining data (Crowther *et al.*, 1985; Craig *et al.*, 1987). They also strengthen the conclusions based on negative staining of other species of myosin filaments that the activation causes loss of relaxed order (Levine, 1986; Ikebe & Ogihara, 1982; Vibert & Craig, 1985) implying that there is a common structural effect on activation (due to binding of Ca^{2+} to the myosin heads, or to phosphorylation of the regulatory light chains). It has recently been possible to detect the helical order of the relaxed state of intact tarantula muscle by using the rapid freezing technique (Granados *et al.*, 1989, 1990); experiments are in progress with tarantula muscle rapidly frozen in the relaxed and phosphorylated state with the aim of directly observing these changes in micrographs of intact tarantula muscle.

In conclusion our results further support the view that the myosin heads are less bound to the filament in the phosphorylated state, which may facilitate in intact muscle the interaction of the myosin heads with the actin molecules during muscle contraction (Craig *et al.*, 1987).

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