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Unfixed Cryosections of Striated Muscle to Study Dynamic Molecular Events

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ABSTRACT The structures of the actin and myosin filaments of striated muscle have been studied extensively in the past by sectioning of fixed specimens. However, chemical fixation alters molecular details and prevents biochemically induced structural changes. To overcome these problems, we investigate here the potential of cryosectioning *unfixed* muscle. In cryosections of relaxed, unfixed specimens, individual myosin filaments displayed the characteristic helical organization of detached cross-bridges, but the filament lattice had disintegrated. To preserve both the filament lattice and the molecular structure of the filaments, we decided to section unfixed rigor muscle, stabilized by actomyosin cross-bridges. The best sections showed periodic, angled cross-bridges attached to actin and their Fourier transforms displayed layer lines similar to those in x-ray diffraction patterns of rigor muscle. To preserve *relaxed* filaments in their original lattice, unfixed sections of rigor muscle were picked up on a grid and relaxed before negative staining. The myosin and actin filaments showed the characteristic helical arrangements of detached cross-bridges and actin subunits, and Fourier transforms were similar to x-ray patterns of relaxed muscle. We conclude that the rigor structure of muscle and the ability of the filament lattice to undergo the rigor-relaxed transformation can be preserved in unfixed cryosections. In the future, it should be possible to carry out dynamic studies of active sarcomeres by cryo-electron microscopy.

INTRODUCTION

Understanding the last secrets of muscle contraction requires that the contractile components be investigated at the molecular level in the native filament lattice. Electron microscopy provides molecular level detail of *isolated* contractile filaments studied by negative staining (e.g., Kensler and Levine, 1982; Kensler et al., 1985; Vibert and Craig, 1983) and by cryoelectron microscopy (Ménétret et al., 1988, 1990; Milligan et al., 1990; Vibert, 1992). Ultra-thin *sectioning* of conventionally fixed and embedded muscle, on the other hand, reveals the filaments in their native lattice arrangement, but fine details of molecular arrangement within the filaments are lost (Reedy, 1976a, b). Molecular preservation is greatly improved in embedded specimens that are fixed first with tannic acid (Reedy et al., 1987, 1991) or are rapidly frozen and then freeze-substituted (e.g., Tsukita and Yano, 1985; Padron et al., 1988; Lepault et al., 1991; Craig et al., 1992; Hirose et al., 1993).

All of these methods involve some kind of chemical fixation and embedding or fragmentation of the muscle before observation. Ideally, one would like to avoid both of these treatments: intact, uncryoprotected specimens would be rapidly frozen, cryosectioned, and the cryosections observed in the frozen-hydrated state. This ideal has been approximated by McDowall et al. (1984) (using 15% sucrose as cryoprotectant), revealing "native" detail of the myosin and actin filaments in the case of insect flight muscle (McDowall et al., 1984). However, such sections are very difficult to obtain and are subject to mechanical distortion during sectioning (McDowall et al., 1984; Trus et al., 1989). Two alternative, easier methods have been developed. The first, developed by Tokuyasu for antibody-labeling purposes, involves cryosectioning of fixed, strongly cryoprotected specimens followed by positive staining (Tokuyasu, 1973, 1980, 1986, 1989). The second method involves negative staining of similarly cryosectioned specimens and reveals considerable ultrastructural detail (Sjöström and Squire, 1977a, b; Sjöström et al., 1991). But again, with both of these methods, fixation may introduce artifacts, and the finest details, such as the arrangement of actin and myosin monomers within the myofilaments, have not been reported. To try to improve fine structural preservation and to offer the possibility of making dynamic experiments, we have studied the effect of omitting chemical cross-linking in the Tokuyasu-Sjöström technique. We conclude that the omission of fixation results in preservation of the structural as well as the biochemical properties of striated muscle.

MATERIALS AND METHODS

Muscle preparation

5-mm-diameter strips of fast striated muscle were dissected from the tail of the lobster, *Homarus americanus* (from a local supermarket) and from the adductor of the scallop, *Placopecten magellanicus* (from the Marine Biological Laboratory, Woods Hole). They were skinned under slight tension for 4 h at 4°C, with regular tumbling, in relaxing buffer (70 mM potassium acetate, 8 mM magnesium acetate, 2 mM EGTA, 4 mM ATP, 0.1 mM NaN_3, 1 mM DTT, 20 mM MOPS, pH 7.0) containing 0.1% saponin and 20 mM, instead of 2 mM, EGTA. At the end of the skinning

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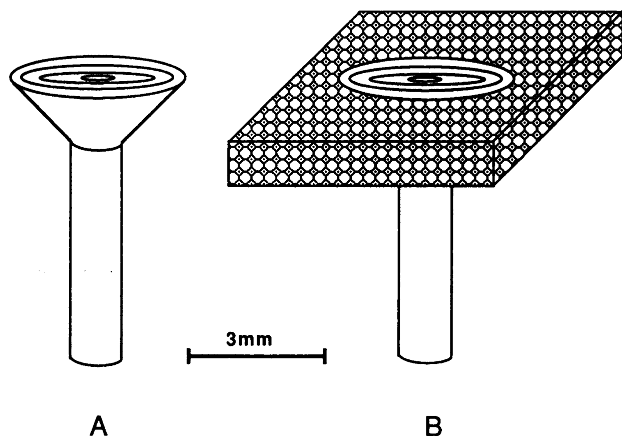

FIGURE 1 Modification of the Reichert cryosection specimen pin (*A*) using a square sheet of polystyrene foam to extend the area of the surface designed to receive the specimen (*B*).

process, the muscle strips were rinsed and stored in relaxing buffer at 4°C until used (within 1 day).

A giant water bug (*Lethocerus*) thorax was provided to us by Dr. Michael Reedy in a 75% glycerol solution containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM ATP, 5 mM NaN_3, 20 mM KPi buffer, pH 6.8. Bundles of about 10 fibers were dissected out and rinsed in relaxing buffer to remove the glycerol.

Cryosectioning

Small bundles (about 1–2 mm in diameter and 15 mm long) of the skinned or glycerinated muscle were slightly stretched (to about body length) and pinned out on silicon rubber sheets (Sylgard, Dow Corning, Midland, MI) at the bottom of petri dishes. The muscle was either kept relaxed or the relaxing buffer was exchanged for rigor buffer (relaxing buffer without ATP) to wash ATP away and induce the rigor state. Some of the bundles were fixed in a solution of 2% glutaraldehyde in the corresponding buffer, pH 7.0, for 1 h at 4°C. The specimens were rinsed for 1 h in buffer to remove the excess of glutaraldehyde before cryoprotection.

The cryoprotectant used was 2.3 M sucrose (Tokuyasu 1973), 2.1 M sucrose + 10% PVP (Tokuyasu, 1989), or 30% glycerol (Sjöström et al., 1991) in the appropriate relaxing or rigor buffer. Bundles were infused with cryoprotectant for 1–2 h at 4°C with gentle tumbling. Fixed muscle bundles could be cut to fit the platform of the Reichert cryosectioning specimen pin (about 3 mm in diameter; Fig. 1 *A*) without distortion of the filament lattice. On the other hand, unfixed muscle bundles, because of their softness, were cut longer (about 10 mm in length) to limit distortion in the region to be sectioned. The specimen pin platform was made larger, to accommodate the longer specimen, by fitting it with a square sheet of polystyrene foam (Fig. 1 *B*), which was removed after freezing and trimming. Muscle pieces on the original or modified specimen pins were quench-frozen in a liquid ethane/liquid propane (25%/75%) mixture cooled to liquid nitrogen temperature (Hirose and Koike, 1991).

The samples were sectioned at −110°C on a dry, 35° Diatome (Fort Washington, PA) cryo-diamond knife or on a glass knife, made according to Tokuyasu and Okamara (1959) on an LKB knifemaker (Leica, Deerfield, IL), using an FC4D or FCS Reichert (Leica) cryo-ultramicrotome and a Diatome antistatic device (Static Line). The cryosections were picked up on electron microscope grids coated with a perforated carbon film (Fukami and Adachi, 1963), which itself supported a thinner (~20 nm) carbon film. Ribbons of *fixed* muscle sections were transferred onto 300 mesh grids in the cryochamber using a platinum loop (2 mm diameter) filled with 2.3 M sucrose as described by Tokuyasu (1973, 1980, 1986). To minimize mechanical damage, ribbons of *unfixed* muscle sections were generally transferred on to the grid in the cryochamber using a white dalmatian hair mounted on a wooden stick. They were then thawed by dropping 20 μl of buffer on to the grid still in the cryochamber and then quickly brought to room temperature. The grids supporting the thawed sections were floated (specimen side down) on about 20 ml of distilled water (for fixed specimen) or buffer at 4°C (for unfixed specimens) for a period of 10 min to wash the cryoprotectant away, and then negatively stained with 2% uranyl acetate. To help the spreading of the stain, in most cases (see figure legends), a square of carbon film (about 20 nm thick) picked up with a 3.5 mm platinum loop from a trough of 2% uranyl acetate was applied to the surface of the grid. The excess of stain was blotted from the edge of the grid. This resulted in the negatively stained sections being "sandwiched" between two carbon films in a uniform film of stain.

Electron microscopy and image analysis

Specimens were observed in a JEOL (Peabody, MA) 100CX electron microscope operated at 80 kV using conventional electron doses. Micrographs were screened using an optical diffractometer (Salmon and DeRosier, 1981) to select the best ordered areas of the sections. The best micrographs were digitized using a Panasonic WV-BD400 CCD camera mounted with a 60 mm Nikon AF MICRO NIKKOR lens. The pixel size corresponded to approximately 1.0 nm in the original specimen except in Fig. 4, where it was 1.5 nm. The digitized image was computationally boxed and processed on a SUN SPARCstation LX (Mountain View, CA) using VAX 11–780 programs from the MRC Laboratory of Molecular Biology image processing package adapted to run under UNIX (kindly provided by Dr Raul Padron; Craig et al., 1992). Scale markers on Fourier transforms indicate the spacings of the corresponding x-ray diffraction reflections (for insect flight muscle: Reedy et al., 1983; Miller and Tregear, 1972; for lobster and scallop muscle: Wray et al., 1975, 1978), not absolute spacings measured from the electron micrographs. Image filtering was performed by computational masking of the layer lines visible in the computed transform (see figure legends).

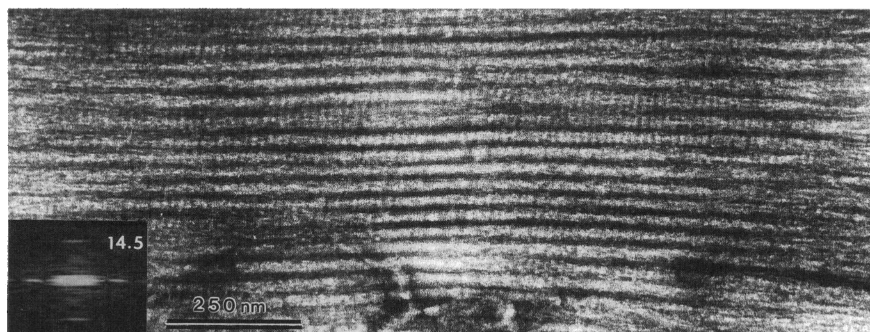

FIGURE 2 Longitudinal cryosection of fixed, relaxed scallop striated adductor muscle, negatively stained with uranyl acetate. Filaments appear light against a dark background. The bare zone of the myosin filaments is responsible for the break in the 14-nm periodicity at the center of the sarcomere. (*insert*) Computed Fourier transform of part of Fig. 1, showing 14.5 nm meridional reflection.

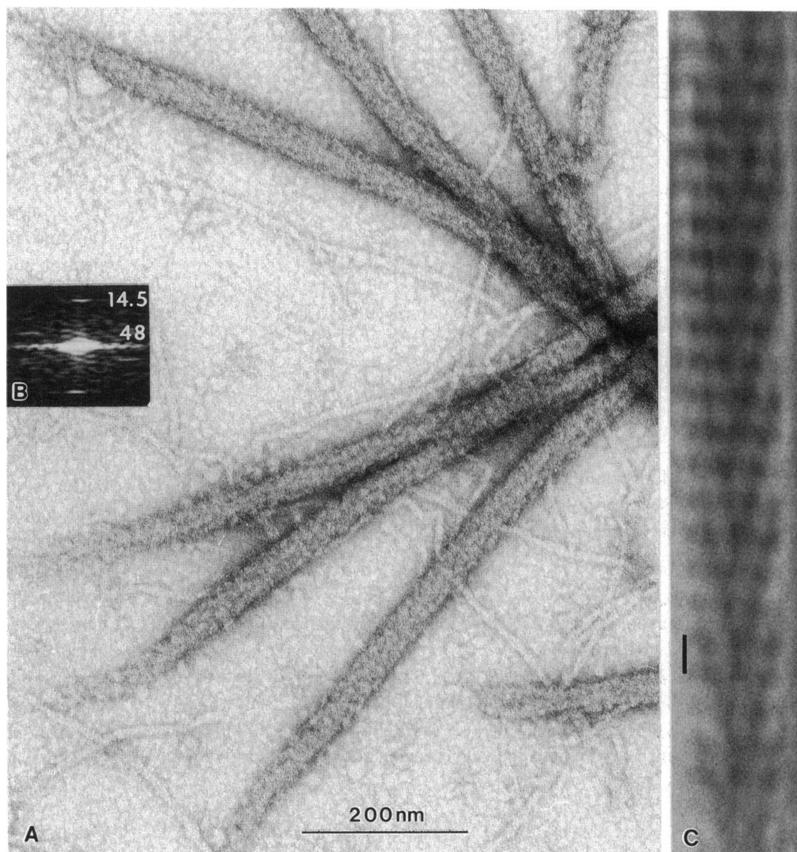

FIGURE 3 (*A*) Dispersed filaments from section of unfixed, relaxed scallop striated muscle, showing helical preservation of myosin crossbridges. (*B*) Computed Fourier transform of a filament from *A* showing layer-lines of spacing 48 and 14.5 nm. (*C*) Filtered image of filament used in *B* using the equator and the 48 and 14.5 nm layer lines extending radially to 1/9.6 nm^{-1}. The contrast is reversed compared with original image in *A*. Scale bar = 14.5 nm.

RESULTS

Relaxed muscle

Fixed relaxed scallop muscle

Scallop striated muscle was studied because its myosin filaments have a well defined helical arrangement that has been well studied by x-ray diffraction and electron microscopic techniques (Wray et al., 1975; Vibert and Craig, 1983; Vibert, 1992). Cryosections of scallop striated muscle fixed in the relaxed state showed a clear 14.5 nm periodicity along the myosin filaments, which was in register across the A-band (Fig. 2), and a corresponding, strong meridional reflection in the Fourier transform (Fig. 2, inset): this repeat arises from the axial spacing of the cross-bridges along the thick filaments. The helical arrangement of the cross-bridges in the myosin filaments and of actin monomers in the thin filaments was not visible.

Unfixed relaxed scallop muscle

Without fixation before sectioning, the native filament lattice was disrupted (Fig. 3 *A*), presumably because the myosin

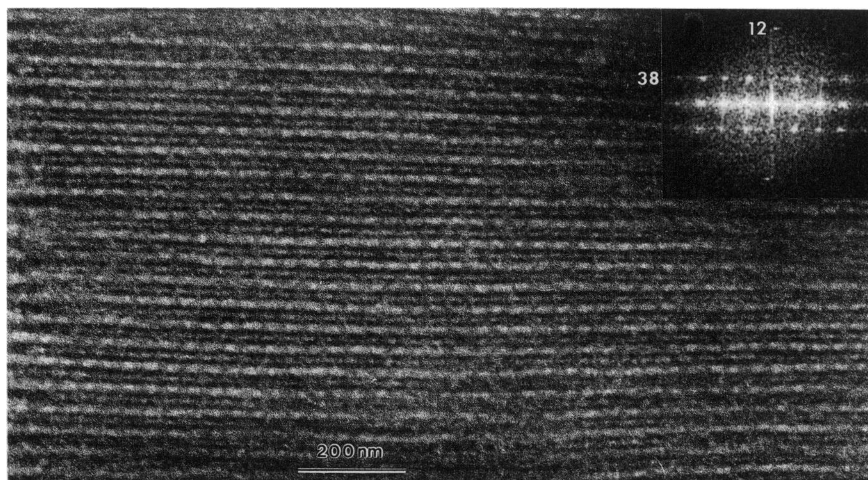

FIGURE 4 Section of lobster muscle fixed in the rigor state showing periodicity consistent with "chevron" structures arising from the angled attachment of the myosin crossbridges to actin filaments. In well ordered specimens, the periodicity is aligned across the A band. (*insert*) Computed Fourier transform showing information along the layer lines of spacing 38, 19, and 12 nm, sampled by the filament lattice.

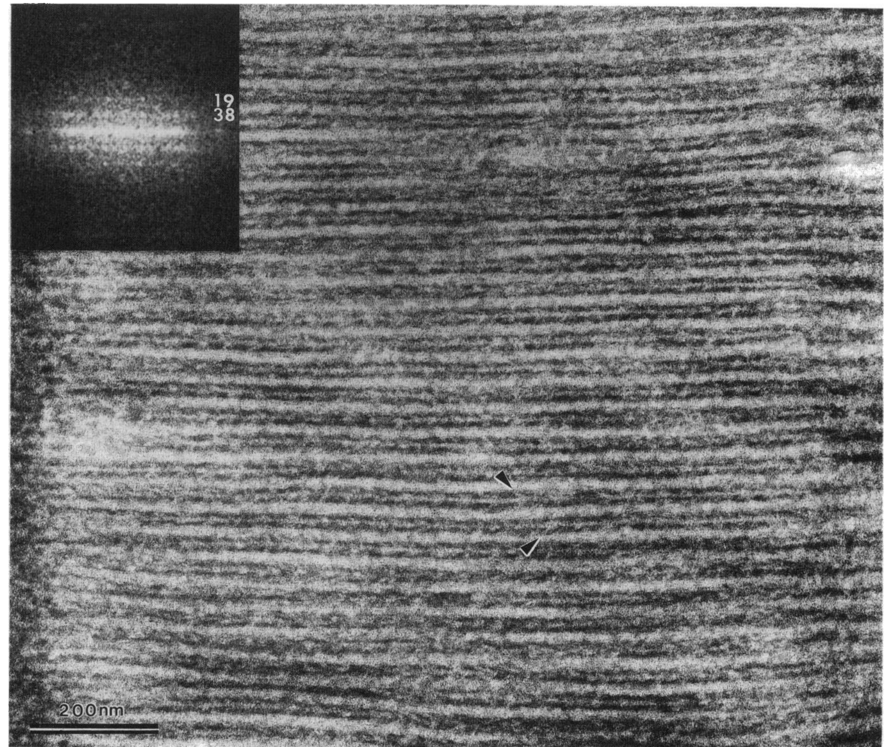

FIGURE 5 Section of insect muscle fixed in the rigor state. Cross-bridge chevrons joining thick and thin filaments are marked with arrows. (*insert*) Computed Fourier transform showing information along the 38 and 19 nm layer lines.

cross-bridges were detached from the actin filaments in the presence of ATP and, thus, the filaments could dissociate from each other when the section thawed. Individual relaxed myosin filaments displayed a clear helical array of cross-bridges in both original (Fig. 3 *A*) and filtered (Fig. 3 *C*) images. Fourier transforms showed a corresponding 14.5 nm meridional reflection and a 48 nm layer line (Fig. 3 *B*), as seen in x-ray diffraction patterns of scallop muscle and in Fourier transforms of electron micrographs of isolated filaments (Wray et al., 1975; Vibert and Craig, 1983).

Rigor muscle

Although sectioning of unfixed relaxed muscle proved a useful method for observing isolated myosin filaments without the strong mechanical forces involved in homogenizing intact muscle (Vibert and Craig, 1983), our goal in this paper was to observe unfixed filaments in their native lattice. We therefore decided to take advantage of the natural crosslinks between actin and myosin filaments that occur in the rigor state, hoping that these links would hold the filaments in their native lattice during section preparation. To test the success of this strategy, we compared the sections with those obtained with rigor muscle covalently cross-linked by fixation. Insect flight muscle and fast lobster muscle were used in these studies because they have a well ordered rigor structure (Reedy, 1968; Wray et al., 1978).

Fixed rigor muscle

Sections of lobster and insect muscle fixed in the rigor state showed periodicity along the thin filaments (Figs. 4 and 5) consistent with the chevron pattern of cross-bridges shown by Reedy (1968). In some cases, individual chevrons were directly visible (Fig. 5, arrows). In well ordered specimens, chevrons were aligned across the A band with a repeat of 38 nm (Fig. 4). That periodicity and sometimes its second order, sampled by the filament lattice, were found in the corresponding Fourier transforms (Figs. 4 and 5, insets). In addition, a reflection corresponding to a 12-nm periodicity was observed (Fig. 4, inset).

Unfixed rigor muscle

In sections of unfixed insect (Fig. 6 *A*) and lobster (Fig. 6 *B*) the chevrons could again be observed, although areas of ordered structure were not as extensive as in fixed muscle. Fourier transforms (insets) showed layer lines of spacing 38 and 5.9 nm in the case of lobster (Fig. 6 *B*, inset) and a more complete pattern in the case of the insect (inset Fig. 6 *A*, inset), containing additional layer lines of spacing 19, 14.5, 12, and 7.2 nm. The top portion of Fig. 6 *A* has been further processed to reveal its periodic features. The image was first digitized and its contrast enhanced (Fig. 7 *B*). A filtered image was then produced (Fig. 7 *A*) using the periodic features observed in the Fourier transform of Fig. 6 *A* (inset). This procedure allows us to visualize crosslinks between actin and myosin in more detail.

Unfixed rigor sections relaxed on the grid

Unfixed cryosections offer the possibility of studying structural changes in cross-bridge organization in response to changes in biochemical conditions.

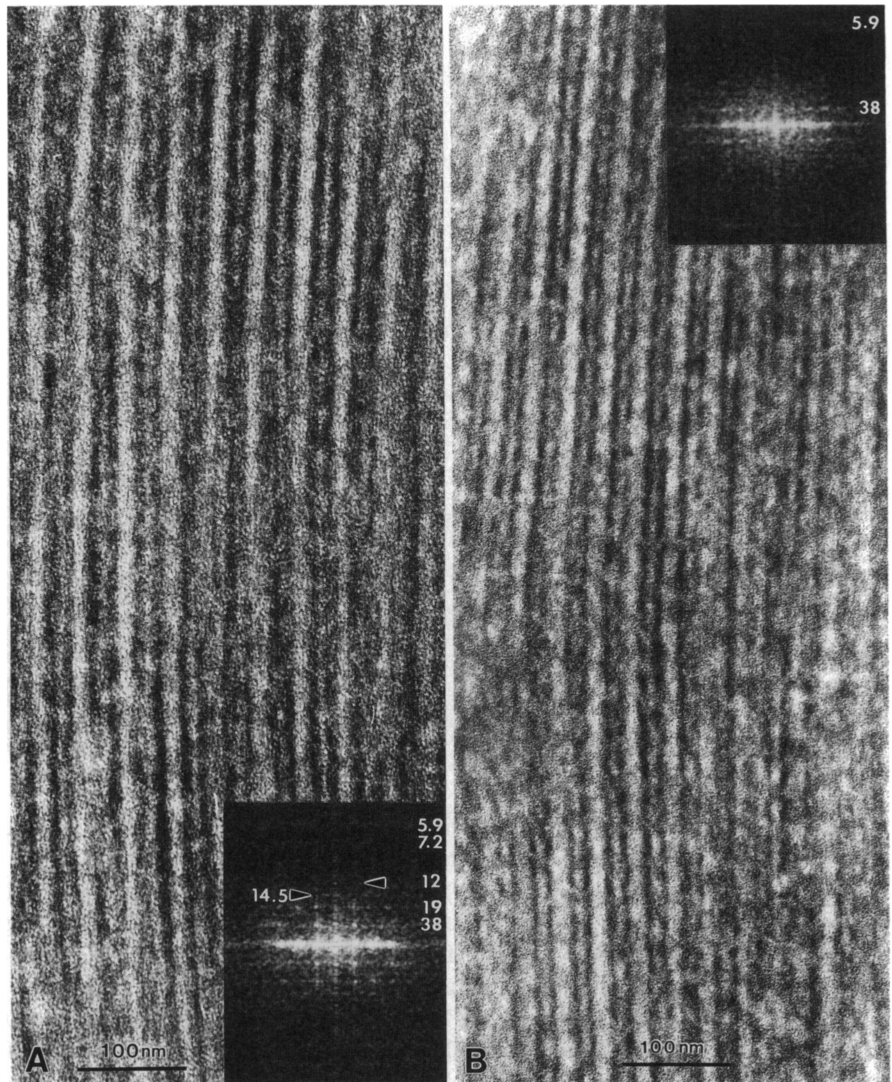

FIGURE 6 Sections of unfixed muscle in rigor. (*A*) Insect flight muscle; (*B*) lobster muscle. (*inserts*) Computed Fourier transforms showing layer-lines of spacing (*A*) 38, 19, 14.5, 12, 7.2, and 5.9 nm, and (*B*) 38 and 5.9 nm.

When sections of unfixed insect muscle in rigor were washed with relaxing buffer before negative staining, most of the cross-bridges became well ordered onto a 14.5-nm repeat, which was in register across the sarcomere (Fig. 8). This repeat was present over large areas even though the initial areas of well ordered rigor structure were small. The changes in periodicity caused by relaxation were reflected in the corresponding Fourier transform (Fig. 8, inset). The 38 nm layer line was weak, and the meridional reflections at 14.5 and 7.2 nm were strong, implying that the cross-bridges had mostly detached from the thin filaments and become ordered on to the thick filament repeat. The 5.9 nm layer line was still visible, whereas the off-meridional reflection at 7.2 nm had disappeared.

DISCUSSION

To test the advantages of studying unfixed muscle by cryosectioning, we have compared both the general appearance and the periodicities of fixed and unfixed sections. We find that omission of the chemical fixation step avoids the artifactual molecular level damage that occurs when proteins are crosslinked by aldehydes and also keeps the contractile apparatus in a "working state." At the same time we find that unfixed sections are more subject to mechanical distortion.

The enhanced preservation of molecular level detail in the absence of fixation is quite clear when the periodicities of fixed and unfixed sections are compared with x-ray diffraction data from intact muscle. In relaxed, unfixed scallop muscle, both helical (48 nm) and axial (14.5 nm) periodicities of the myosin heads around the thick filament are preserved (cf. Wray et al., 1975), whereas fixation appears to destroy the helical ordering. In rigor insect muscles that have not been fixed, the periodic attachment of cross-bridges to actin filaments is preserved, including the 38 and 19 nm layer lines, coming from the long-pitch actin helices, and the high order actin-based reflections at 7.2 and 5.9 nm, from the actin genetic helix (cf. Reedy et al., 1965; Miller and Tregear, 1972). After fixation, the high order reflections disappear. In the case of lobster, fewer reflections are seen, but fixation is still observed to have a similar deleterious effect.

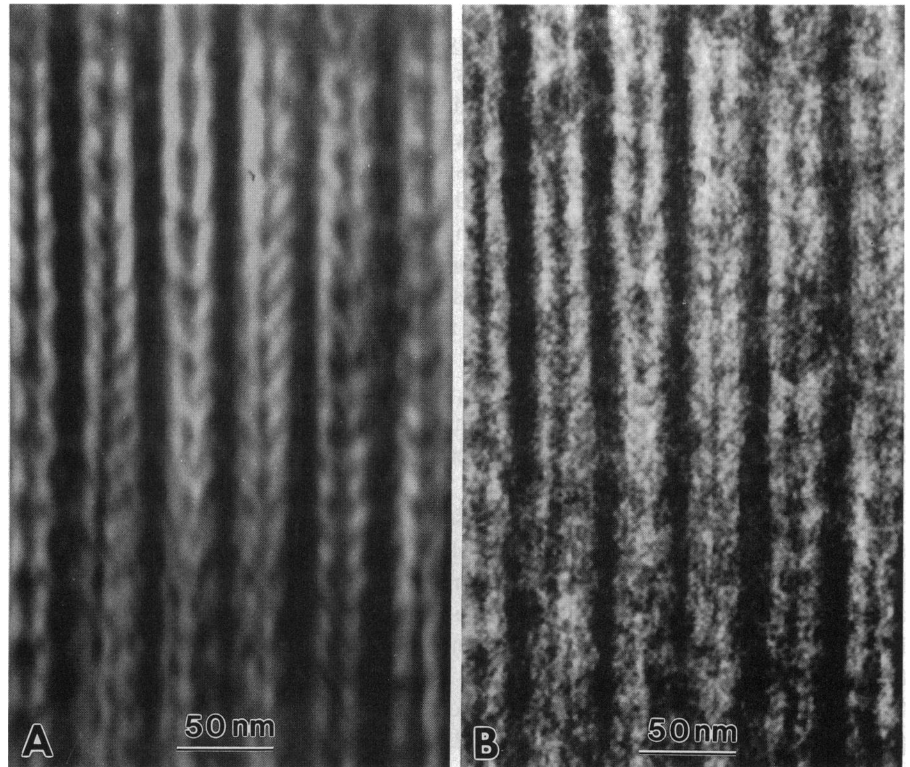

FIGURE 7 Image processing of Fig. 6 *A*. (*A*) Filtered image using equator, 38, and 19 nm layer lines of Fig. 6 *A*, extending radially to 1/7.4 nm^{-1}. This image shows angled cross-bridges attached to actin filaments; (*B*) original image after digitization and contrast enhancement by histogram expansion. Contrast in both is reversed compared with Fig. 6 *A* so that the filaments appear black.

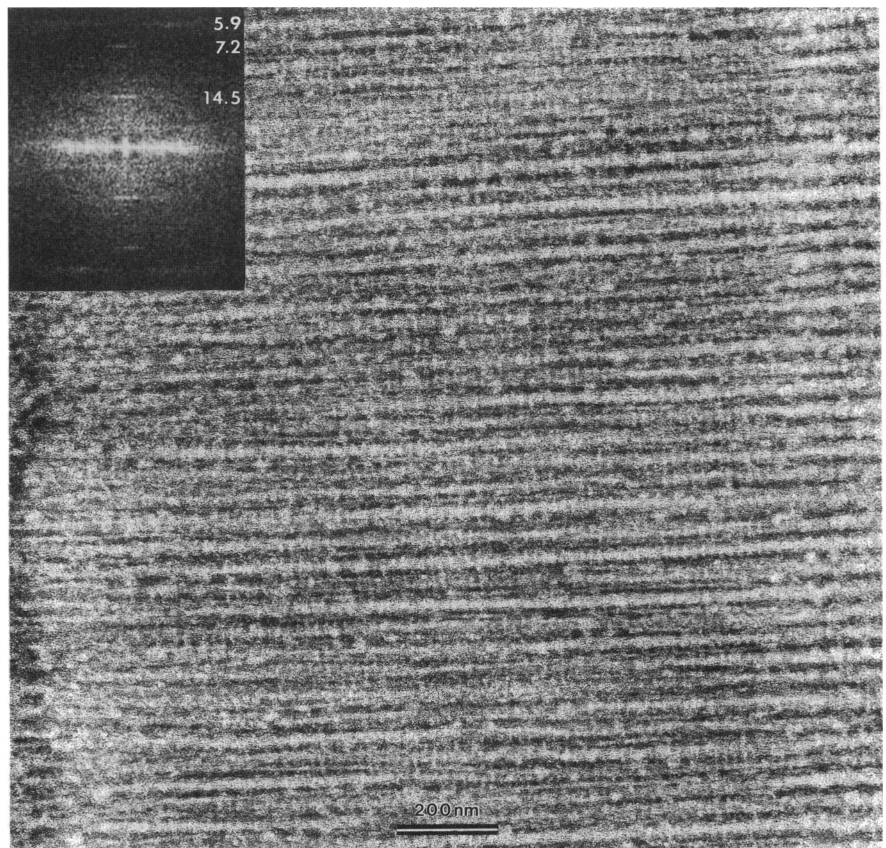

FIGURE 8 Unfixed section of rigor insect flight muscle relaxed on the grid before staining, by rinsing with relaxing solution. (*insert*) Computed Fourier transform showing layer-lines of spacing 14.5, 7.2, 5.9 nm.

The fact that the contractile apparatus of unfixed specimens remains in a "working" state is clearly demonstrated by the observation that rinsing rigor sections with relaxing solution results in transformation of the sections to the relaxed structure. The low order actin-based layer lines, caused mainly by the attachment of myosin heads to the actin helix, virtually disappear while meridional reflections appear at 14.5 and 7.2 nm, reflecting an axial ordering of myosin heads on to the thick filament backbone. In some cases, a weak layer line is still observed at 38 nm, possibly reflecting a helical component to the ordering of the myosin heads (cf. Miller and Tregear, 1972) or incomplete relaxation. In addition, the 5.9 nm layer line is retained, now presumably because of actin monomers mostly unmarked by myosin heads; the unmarked long-pitch actin helices might also contribute to the residual 38 nm layer line.

The absence of covalent (aldehyde) crosslinking has a significant impact on the integrity of the filament lattice, allowing dispersal of actin and myosin filaments that are not attached to each other by any other means. Thus, when unfixed relaxed muscle is cryosectioned and negatively stained, the filaments fall apart from each other, presumably after thawing of the sections. This could be used as a method of preparing separated filaments, which avoids the strong forces typically occurring during tissue homogenization. When noncovalent crosslinks (rigor cross-bridges) are induced between actin and myosin filaments before freezing, forming a three-dimensional filament network, the filament lattice holds together through sectioning, thawing, and staining, although the small size of the well ordered area suggests mechanical distortion. Such distortion is still the main concern of the technique since sections thin enough for useful interpretation are very vulnerable to mechanical stress. Nevertheless, we have shown that sections of unfixed muscle are still capable of physiologically relevant biochemical and structural changes. Such sections could potentially be used to observe structural changes in rapidly activated sections by either negative staining (Frado and Craig, 1992) or cryo-electron microscopy (Ménétret et al., 1991). They are also likely to be of value in antibody labeling studies of tissues containing fixation-sensitive antigens.

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