Mammalian Cardiac Muscle Thick Filaments: Their Periodicity and Interactions with Actin

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ABSTRACT Cardiac muscle has been extensively studied, but little information is available on the detailed macromolecular structure of its thick filament. To elucidate the structure of these filaments I have developed a procedure to isolate the cardiac thick filaments for study by electron microscopy and computer image analysis. This procedure uses chemical skinning with Triton X-100 to avoid contraction of the muscle that occurs using the procedures previously developed for isolation of skeletal muscle thick filaments. The negatively stained isolated filaments appear highly periodic, with a helical repeat every third cross-bridge level (43 nm). Computed Fourier transforms of the filaments show a strong set of layer lines corresponding to a 43-nm near-helical repeat out to the 6th layer line. Additional meridional reflections extend to at least the 12th layer line in averaged transforms of the filaments. The highly periodic structure of the filaments clearly suggests that the weakness of the layer lines in x-ray diffraction patterns of heart muscle is not due to an inherently more disordered cross-bridge arrangement. In addition, the isolated thick filaments are unusual in their strong tendency to remain bound to actin by anti-rigor oriented cross-bridges (state II or state III cross-bridges) under relaxing conditions.

INTRODUCTION

Cardiac muscle is the tissue responsible for the contraction and production of force in the heart. This function involves the interaction between two sets of microscopic filaments: the myosin-containing thick filaments and the actin-containing thin filaments. Although similar to skeletal muscle in its arrangement of thick and thin filaments into sarcomeres, cardiac muscle differs from skeletal muscle in a number of physiological properties, including its high resting tension, steep relationship between active force and sarcomere length (Frank-Starling relation: Fuchs and Wang, 1997), and modulatory regulation by phosphorylation (Jeacocke and England, 1980; Hartzell and Titus, 1982; Garvey et al., 1988; Schlender and Bean, 1991). At least some of these differences may relate to the structure of the cardiac thick filament and its cross-bridges (Matsubara and Millman, 1974; Weisberg and Winegrad, 1998) or accessory proteins (Weisberg and Winegrad, 1998; Jeacocke and England, 1980; Hartzell and Titus, 1982; Garvey et al., 1988; Schlender and Bean, 1991). However, despite the importance of cardiac muscle and the extensive literature on its general structure and characteristics (Bers et al., 1989; Forbes and Sperelakis, 1983; Miller et al., 1985; Miller and Smith, 1985; Sommer and Jennings, 1986; Linke et al., 1994; Cannell et al., 1994; Martin and Barsotti, 1994), very little information is available on the detailed molecular structure of the cardiac thick filament.

One approach to elucidating the structure of muscle filaments has been by x-ray diffraction. However, only a few x-ray diffraction studies have been performed on cardiac muscle. Although these studies demonstrated that the heads in the relaxed muscle are near-helically arranged with a helical repeat every 43 nm, as in skeletal muscle (Matsubara and Millman, 1974; Matsubara et al., 1989), the layer lines in the x-ray diffraction patterns from heart were very weak compared with the patterns from skeletal muscle (Matsubara and Millman, 1974; Matsubara et al., 1989). It was not clear from these studies whether the weakness of the patterns was due to inherent differences in the degree of order in the arrangement of the myosin heads in cardiac muscle compared with skeletal muscle, or from other causes (Matsubara et al., 1989). In addition, although providing evidence that ~20% of the heads under relaxing conditions are associated with actin in a non-force-producing conformation, these studies did not allow the exact nature of that interaction to be determined (Matsubara et al., 1989).

Another approach to the problem of elucidating the structure of the cardiac muscle thick filament is by using electron microscopy and computer image analysis developed for the skeletal muscle thick filaments. General electron microscope studies have shown the thick filament to have a bipolar structure, with the myosin heads arrayed on the arms of the filament (Huxley, 1963; Craig and Offer, 1976; Trinick and Elliot, 1979). Although these early papers did not show the expected helical arrangement of the myosin heads, we have more recently shown that the helical order can be preserved in isolated negatively stained filaments, and computer image analysis has been used to show evidence for a three-stranded arrangement of the myosin heads in filaments of the frog (Kensler and Stewart, 1983, 1986; Stewart and Kensler, 1986), fish (Kensler and Stewart, 1989), rabbit (Kensler and Stewart, 1993), and chicken (Kensler and Woodhead, 1995). Additional electron micro-

Submitted April 30, 2001, and accepted for publication November 26, 2001.

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0006-3495/02/03/1497/12 $2.00

scopic evidence for the helical arrangement of the myosin heads has also come from studies of freeze-fracture deep-etched muscle (Variano-Marston et al., 1984), freeze-substituted muscle (Padron and Craig, 1989; Craig et al., 1992), and frozen-hydrated filaments (Menetret et al., 1990). In the case of isolated negatively stained frog filaments we have calculated a three-dimensional reconstruction of the thick filament that clearly shows the three-stranded arrangement of the myosin heads and the presence of an axial, azimuthal, and radial perturbation of the heads from an ideal helical arrangement (Stewart and Kensler, 1986).

As a first step in this direction I describe a method for isolation of cardiac muscle thick filaments that allows the structure to be analyzed with a combination of electron microscopy and computer image analysis. I demonstrate that these filaments have a highly periodic arrangement of the myosin heads, and that the weakness of the x-ray diffraction patterns is not due to inherent disorder in the cross-bridge arrangement. In addition, I present evidence that these filaments have a greater tendency to interact with actin filaments under relaxing conditions than do skeletal muscle thick filaments, thus supporting the x-ray diffraction data of Matsubara et al. (1989), and potentially providing a system for the structural study of weakly binding cross-bridges.

MATERIALS AND METHODS

Filament isolation

Thick filaments were isolated from rabbit papillary muscle with a modification of previously described procedures for the isolation of vertebrate skeletal muscle thick filaments (Kensler and Stewart, 1983, 1986, 1989; Kensler et al., 1994). Papillary muscles were rapidly dissected from rabbit hearts and placed into two to three changes (15 min each) of an EGTA-relaxing solution containing 100 mM NaCl, 2 mM EGTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 2.5 mM ATP to remove any excess extracellular calcium. The muscles were then teased with forcep tips into fine bundles 0.5 mm or less in diameter, and placed for 1 h with stirring into a relaxing solution of the same composition, but containing 1.0% Triton X-100 to chemically skin the muscle (Magid and Reedy, 1980), thus making the muscle permeable to the relaxing solution. The muscle was rinsed with two to three changes of fresh relaxing solution without Triton to remove any residual detergent.

Separation of the filaments was accomplished with the elastase treatment described by Levine et al. (1996), a modification of the original procedure of Magid et al. (1984). In this procedure, a small piece of the muscle was incubated in an elastase solution (1.1 mg/ml elastase in relaxing solution) containing the proteolytic inhibitor cocktail (0.44 mg/ml trypsin inhibitor, 0.0004 mg/ml pepstatin A, 0.0004 mg/ml leupeptin, 0.0004 mg/ml aprotinin, and 0.04 mM PMSF) of Sellers (1981) for 3 min, and then transferred to an Eppendorf tube containing fresh relaxing solution (with the proteolytic inhibitors at 2 mg/l). This was vigorously shaken by hand for 3 min and then gently pipetted up and down in a disposable pipette, breaking up the muscle and yielding a suspension of separated thick and thin filaments, which was then examined by electron microscopy.

Negative staining

Negative staining was performed by the tannic acid-uranyl acetate procedure previously described (Kensler et al., 1985). In this procedure, thick filaments were absorbed either onto thin carbon films (5–7-nm thickness) supported on perforated Formvar-coated grids or directly onto perforated Formvar-coated grids (without the supporting thin carbon film). These were rinsed sequentially with eight drops each of 1) half-strength relaxing solution; 2) 0.25% tannic acid (Mallinckrodt AR 1764, Mallinckrodt, Inc., St. Louis, MO) in half-strength relaxing solution; 3) half-strength relaxing solution again; and 4) 1% uranyl acetate. The half-strength relaxing solution contained 50 mM KCl, 1 mM EGTA, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM ATP, and 5 mM imidazole buffer at pH 7. All staining on these filaments used rinse and stain solutions maintained at 25°C on a Thermolyne Dri-bath heater to avoid the loss of helical ordering that occurs in mammalian and avian thick filaments at temperatures below ~15°C (Wray, 1987; Wakabayashi et al., 1988; Kensler et al., 1994; Kensler and Woodhead, 1995).

Electron microscopy

Electron microscopy of the negatively stained preparations was performed at 80 kV with a JEM-1200EXII electron microscope (JEOL U.S.A. Electron Optics, Peabody, MA) or a Philips EM200 (Philips Electronic Instruments, Mahwah, NJ). Micrographs were recorded at either 20,000 or 40,000× for analysis by optical diffraction or computed Fourier transforms. Magnification was calibrated using catalase crystals (Wrigley, 1968). Irradiation of the sample was reduced by searching the grid at very low magnifications (5000× or less) under minimal irradiation conditions. Exposure to the beam at higher magnifications was minimized to only that necessary for exposure of the film.

Assessment of filament periodicity

Filament periodicity was assessed either by optical diffraction or from computed Fourier transforms. Optical diffraction was performed as previously described (Kensler and Levine, 1982; Kensler and Stewart, 1983, 1986; Kensler et al., 1985) using a folded beam helium neon laser diffractometer with a focal distance of ~3 meters. The diffraction constant for the diffractometer was calculated using a grating with 0.1 mm spacings. The spacings on the diffraction patterns were calculated either using this diffraction constant in conjunction with the electron microscope magnification, or were normalized relative to the spacing (1/14.3 nm^{-1}) of the meridional reflection on the third layer line (Wray, 1982).

For computation of Fourier transforms, selected areas of filament images recorded at 40,000× were densitometered with a Nikon LS1000 Slide Scanner (Nikon Corporation, Melville, NY) at a pixel size corresponding to ~0.8 nm. The measured gray values were linearized and converted to optical density values before floating the boxed image areas (60 × 512 pixels) in 512 × 512 arrays. Apodization of the edges of the boxed images was performed as previously described (Kensler and Stewart, 1983; Stewart et al., 1985; Stewart and Kensler, 1986; Stewart, 1988) to reduce the effect of the edges of the box on the computed transform. When necessary, the images were computationally unbent as described by Bremer et al. (1991), rescaled and rotated so the helical layer lines fell on the sampled lines of the transform. The calculations were performed on a 233 MHz Pentium II and the results displayed as gray scale images.

RESULTS

Isolation procedure

Although thick filaments are easily isolated from skeletal muscle without detergent skinning (Kensler and Stewart,

1983, 1986, 1989, 1993; Kensler et al., 1994), attempts to use these procedures with cardiac muscle resulted either in the release of only a few filaments or of fragments. Cardiac muscle, incubated overnight in relaxing solution, appeared to undergo extensive contraction, thus limiting the release of the filaments. Attempts to overcome this problem by increasing the concentration of EGTA or ATP, soaking longer in the relaxing solution, changing the major anion (chloride or acetate), or changing the force or type of homogenization appear to have little effect. However, chemically skinning the muscle with 1.0% Triton X-100 before soaking the muscle overnight in relaxing solution alleviates this problem. Using the procedure described here, large numbers of cardiac muscle thick filaments can be isolated (Fig. 1, *A* and *B*).

Isolated filament appearance

Similarly to skeletal muscle thick filaments (Kensler and Stewart, 1986, 1993), the cardiac filaments appear distinctly bipolar with a central bare zone, a length of ~1.6 μm, and a diameter of 31.05 $\pm$ 1.55 nm (n = 100). Many of the filaments at low magnification (Fig. 1, *A* and *B*) appear highly periodic with a well-ordered myosin cross-bridge arrangement. At higher magnification (Fig. 2, *A–D*) this periodic appearance can be seen to arise from a near-helical arrangement of cross-bridges with an axial repeat every third cross-bridge level (*arrows*) consistent with the 43-nm helical repeat (3 $\times$ 14.3 nm) observed by x-ray diffraction (Matsubara et al., 1989). This periodicity can be seen particularly well by tilting the micrograph plate and sighting along the axis of the filaments. Close inspection of the cross-bridge pattern (Fig. 2, *A–C*) reveals that it lacks the mirror plane symmetry across the long axis of the filament, which would be expected if the cross-bridges lie along an even number of helical strands. This is shown particularly well at very high magnification (Fig. 2 *D*) where cross-bridges (*arrowheads*) can often be seen to extend from the filament backbone on one side of the filament, without mirror-image cross-bridges extending from the other side of the filament. This cross-bridge pattern appears qualitatively similar to those we have previously shown for vertebrate skeletal muscle thick filaments (Kensler and Stewart, 1983, 1986, 1989, 1993; Kensler et al., 1994) and is consistent with an odd-stranded symmetry.

Periodicity of the filaments

The periodicity of the cross-bridge regions of the isolated cardiac muscle thick filaments has been confirmed by both optical diffraction and computed Fourier transforms. The computed Fourier transforms (Fig. 3) show a strong series of layer lines indexing close to the expected orders of a 43-nm near-helical arrangement of the cross-bridges. These patterns frequently extend to the 11th or 12th layer line. The meridional reflections on the 3rd and 6th layer lines that correspond to the average axial rise of 14.3 nm between cross-bridge levels (Huxley and Brown, 1967; Matsubara et al., 1989) were of variable intensity between the patterns, as previously found for both rabbit skeletal and frog skeletal muscle thick filaments (Kensler and Stewart, 1983; Kensler et al., 1994). Additional meridional reflections, not expected from ideal helical symmetry, were typically present on the 1st, 2nd, 4th, 5th, 10th, and 11th layer lines.

The arrangement of the diffraction maxima along a distinct set of layer lines corresponding to orders of an axial spacing of 43 nm was particularly evident upon averaging the transforms from several filaments (Fig. 3, *bottom*). In the averaged pattern, the majority of the intensity was confined to the first six layer lines with strong first and second layer lines, and relatively strong third, fourth and fifth layer lines. However, the presence of additional meridional reflections to the 12th layer line (3.6 nm) was very evident. The presence of the meridional reflections to at least the 11th layer line was confirmed in optical diffraction patterns as well, thus making it unlikely that the reflections are an artifact of the image processing. Overall, these results confirm the very strong periodicity of the cross-bridge arrangement in the isolated cardiac thick filaments.

Interactions of the relaxed isolated thick filaments with actin

Compared with skeletal muscle thick filaments (Kensler and Stewart, 1983, 1989; Kensler et al., 1994; Kensler and Woodhead, 1995), rabbit cardiac filaments show a significantly greater tendency to remain attached to adjacent actin thin filaments when isolated (Fig. 4, *A–C*). Up to ~20% of the thick filaments appear to be complexed to actin when first isolated. Because the filaments were isolated under relaxing conditions, this result was not expected. In some cases, thick filaments have two or more bound actins (Fig. 4 *A*). These thick filament-thin filament complexes (Fig. 4, *A–C*) are remarkable in showing a thick filament with most of its cross-bridges in the relaxed helical arrangement, while a fraction of the cross-bridges make contact with the adjacent actin thin filament. Because the thick filaments retain their highly ordered 43-nm relaxed pattern of cross-bridges, they are unlikely to represent filaments with cross-bridges in a rigor conformation. Also, in many places (*arrows* Fig. 4, *B* and *C*), the attached cross-bridges appear angled away from the bare zone with the actin contact point at the more *z*-band-oriented end, and thus in an anti-rigor conformation. Although the origins of the cross-bridges on the thick filament appear to have a spacing of ~43 nm, in some cases (Fig. 4 *B*) it can be seen that the attachment to actin occurs near equivalent points along the actin double helix and, thus, near the expected 38-nm spaced target zones. The attachments appear to have a pattern of two or three cross-

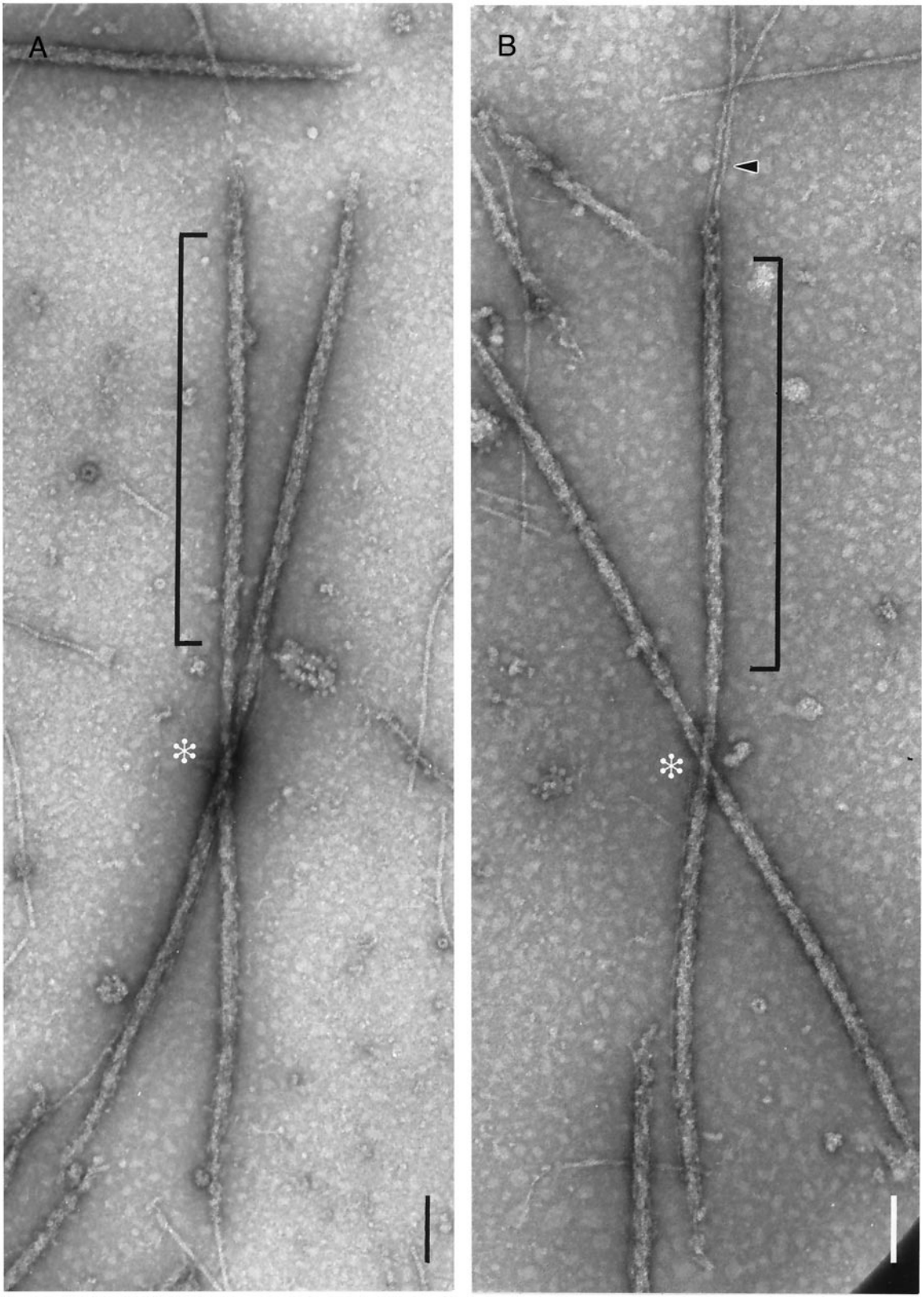

FIGURE 1 Medium magnification electron micrograph fields of isolated and negatively stained rabbit cardiac muscle thick filaments. The brackets indicate regions in which the periodic appearance of the filaments can be particularly well seen. Note that the bracketed filament in *B* has bound actin filaments along it, but still retains the relaxed cross-bridge order. The asterisks indicate the region of the M-band material at the center of the bare zone. Bars = 100 nm.

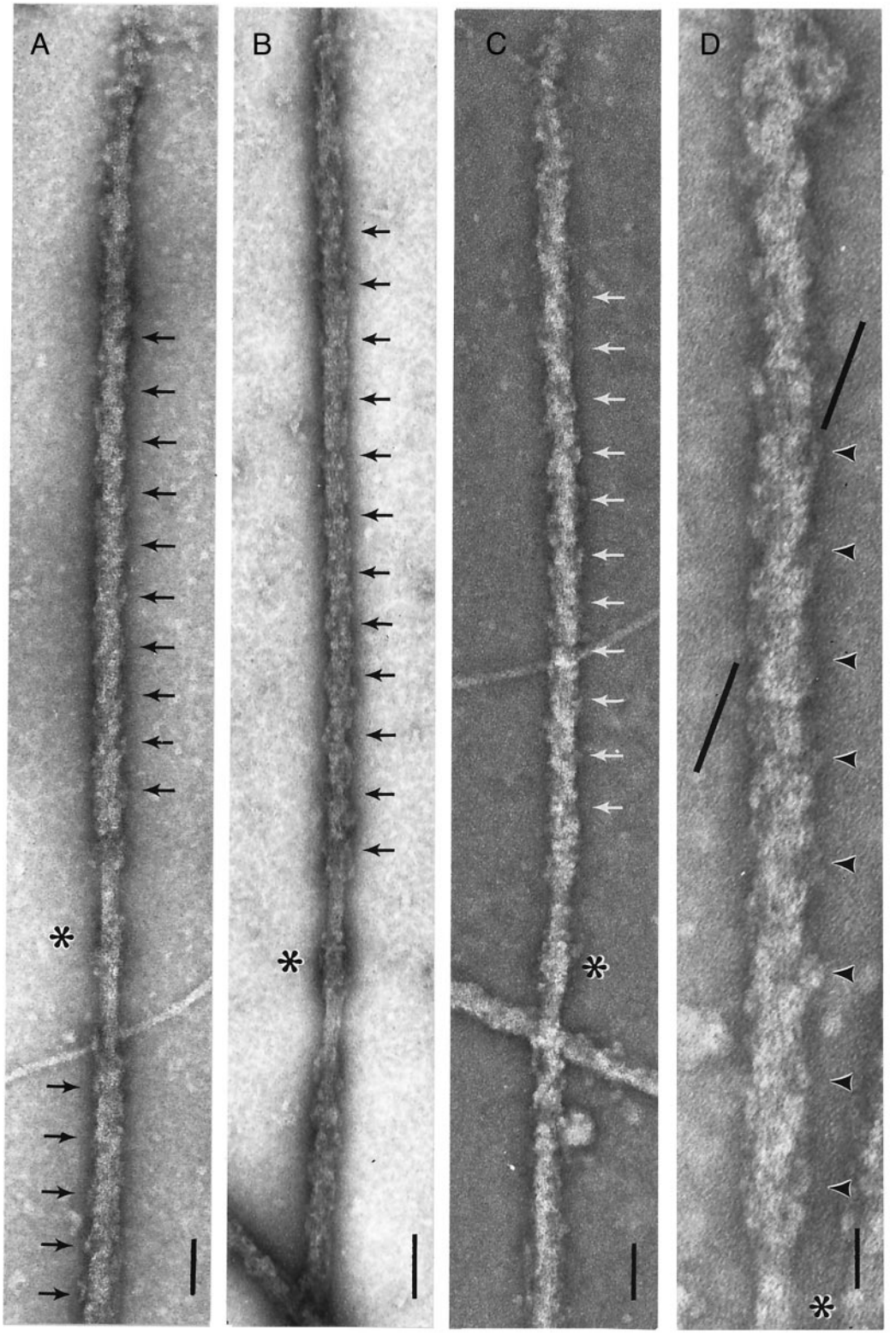

FIGURE 2 High magnification images of the negatively stained rabbit cardiac thick filaments. The arrows in *A–C* and the arrowheads in *D* indicate the distinct 43-nm helical repeat of the filaments. This can best be seen by tilting the figure and sighting along the axis of the filaments. The angled line in *D* indicates the angle of the helical path of the cross-bridges. The lack of mirror plane symmetry of the cross-bridge array across the long axis of the filament is particularly evident in micrograph *D*, where cross-bridges (*arrowheads*) can often be seen projecting from the backbone on the one side of filament, but not on the other side of the backbone at the same cross-bridge level. This clearly indicates the probable odd-stranded structure of the filament. Bars on *A–C* = 50 nm; the bar on *D* = 25 nm.

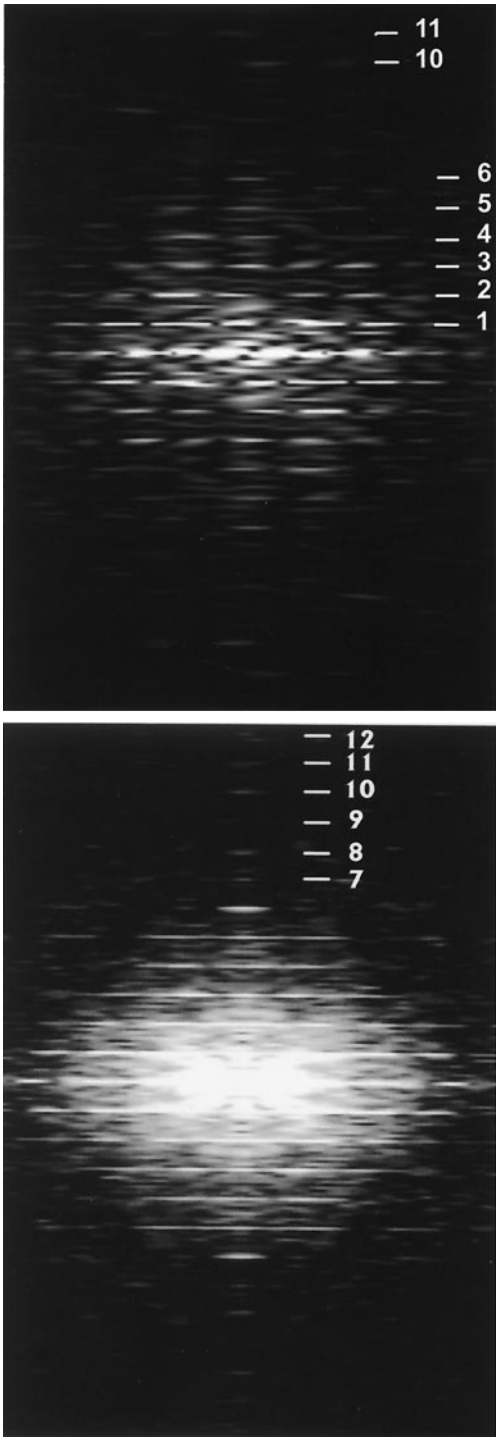

FIGURE 3 A Fourier transform from a region of a single isolated cardiac filament (*top panel*) and a transform representing the average of the transforms from six different filaments (*bottom panel*). The transforms show the set of layer lines corresponding to the 43-nm axial repeat of the near-helical arrangement of the cross-bridges on the filaments. Note the strong first six layer lines in each pattern and the frequent presence of relatively strong "forbidden meridional" reflections on the 8th, 10th, and 11th layer lines.

bridge contacts with the actin, followed by a break in the pattern, and then a cluster of attachments again. This may be consistent with the mismatch between the ~43 nm axial periodicity of the thick filament and the ~38 nm periodicity of the actin thin filament.

DISCUSSION

Filament isolation

As shown above, I developed an isolation procedure for rabbit cardiac muscle thick filaments that preserves the highly periodic near-helical arrangement of cross-bridges in the relaxed filaments. The procedure depends upon chemical skinning of the muscle with Triton X-100. In a few cases filaments were released without chemical skinning, but this was rare. Without chemical skinning, the muscle typically slowly contracts over a period of time into small tough chunks, despite immersion in relaxing solution. Elastase treatment or mechanical homogenization of the muscle in these cases either failed to release filaments or produced fragments. Although this result is different from previous results with various skeletal muscles (Kensler and Stewart, 1983, 1989; Kensler et al., 1994; Kensler and Woodhead, 1995), it is consistent with the results of Miller et al. (1985). In their physiological studies of rat ventricular muscle (Miller and Smith, 1985), they found that the nonskinned muscle in EGTA relaxing solution may show a prolonged slow contraction over a period of hours before slowly falling to a resting tension level somewhat higher than that found after detergent skinning. In contrast, chemical skinning with detergent gave fully relaxed preparations freely permeable to the exogenous ATP. Thus, soaking in an EGTA relaxing solution alone does not appear to allow full relaxation of the cardiac muscle.

In my experiments neither an increase in the concentration of EGTA from 2 mM to 10 mM or an increase in the concentration of ATP from 2.5 mM to 5 mM in the relaxing solution appeared to increase the number of filaments released or their periodicity, nor did an increase in the ATP concentration prevent the slow contraction of the muscle in relaxing solution that occurred when the muscle was left overnight in fresh relaxing solution. To prevent this contraction, it was necessary to skin the muscle in a detergent (Triton X-100) within a few hours of its removal from the animal.

These studies suggest that in cardiac muscle, maintenance of a relatively high concentration of ATP in the external relaxing solution bathing the muscle is not sufficient to stop the contraction of the muscle. The effectiveness of the detergent skinning in allowing the relaxed state to be maintained suggests that there may be a difference in the ability of the isolated cardiac bundles to synthesize new ATP in situ compared to skeletal muscle.

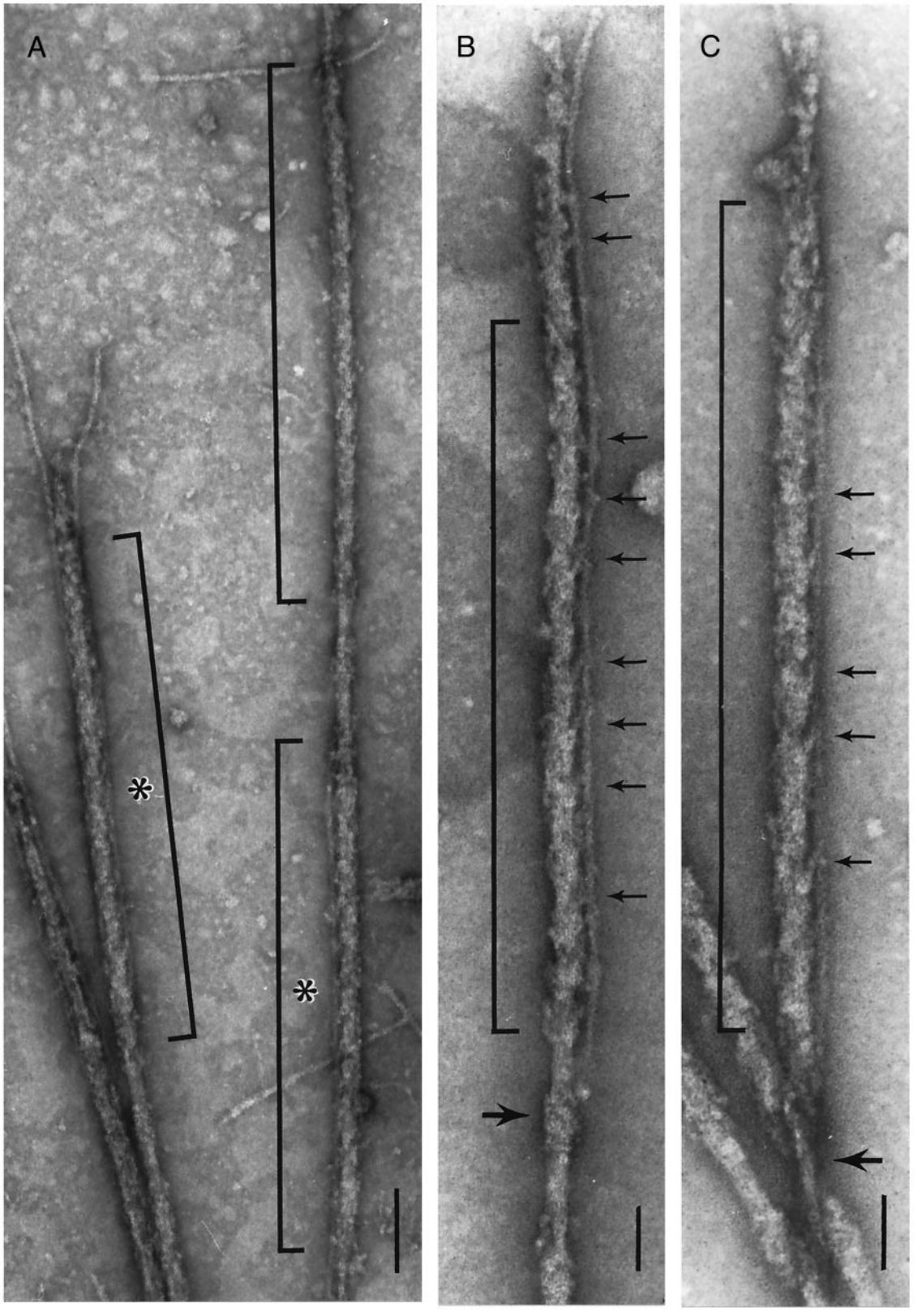

FIGURE 4 Micrographs of the negatively stained cardiac filaments with bound actin thin filaments. (*A*) A medium magnification field in which several of the filaments have bound actin (*brackets*). (*B* and *C*) High magnification images in which the relatively ordered cross-bridge contacts with the filament can be seen in many regions (*brackets*). Note that many of these cross-bridges (*arrows*) appear to have a nonrigor conformation and are angled away from the bare zone at the bottom of the figure. Bars: $A = 100$ nm; B and $C = 5$ nm.

I have also examined the ability of Triton X-100 skinning to "rescue" cardiac muscle that contracted during overnight storage in relaxing solution. Although such detergent treatment of the stored muscle gave a slight increase in the numbers of filaments released compared to the nondetergent-treated muscles, it was clear that for optimal results the detergent skinning must occur within a short period (3 h) after the muscle is excised from the animal. The most likely explanation is that the nonskinned muscle, although bathed with an exogenous relaxing solution, is irreversibly damaged and goes into rigor, and that this damage persists and prevents relaxation when the muscle is "skinned" and exposed to fresh ATP at a later time. To prevent the damage, the muscle must be skinned while still in a relaxed state, thus allowing access of the ATP of the relaxing solution to the myosin heads, and preventing a rigor contraction.

It must be noted that even with the current procedure, the success rate in obtaining release of the cardiac thick filaments in large numbers is not as good as for preparations of the skeletal muscle thick filaments run in parallel. With the described procedure, however, my ability to routinely obtain populations of unbroken ordered cardiac thick filaments has increased to the point that analysis is feasible.

Periodicity of the relaxed cardiac thick filament

Although earlier x-ray diffraction studies of cardiac muscle demonstrated that the heads in the relaxed muscle are near-helically arranged with a helical repeat every 43 nm, as in skeletal muscle (Matsubara and Millman, 1974; Matsubara et al., 1989), the weakness of the layer lines in the patterns from heart (Matsubara and Millman, 1974; Matsubara et al., 1989) left open questions as to the degree of order of the myosin heads in cardiac muscle compared with skeletal muscle. The results presented here demonstrate that the weakness of the x-ray diffraction patterns from relaxed cardiac muscle is not due to an inherent lack of order in the relaxed cross-bridge arrangement. Both the highly periodic images of the filaments and the Fourier transforms derived from them demonstrate that the relaxed rabbit cardiac thick filaments, like skeletal muscle thick filaments, have a very strong 43-nm near-helical periodicity. The computed transforms of the cardiac filaments are very strong and show a series of layer lines corresponding to a 43-nm helical (or near-helical) periodicity with an average axial rise of 14.3 nm between cross-bridge levels. The patterns appear similar to those from isolated rabbit skeletal muscle filaments (Kensler and Stewart, 1993) and are consistent with the x-ray diffraction patterns obtained from vertebrate skeletal muscle (Huxley and Brown, 1967) and mammalian cardiac muscle (Matsubara and Millman, 1974; Matsubara et al., 1989). This suggests that the native structure is well preserved in the isolated cardiac thick filaments.

These results imply that the weakness of the x-ray diffraction patterns must derive from other factors, such as the branching of the muscle fibers or interactions of the heads with actin. Unlike skeletal muscle, in which the fibers within a bundle lie essentially parallel, cardiac muscle shows an extensive pattern of fiber branching. This means that the thick filaments will not all have the same orientation in the muscle, and this could be expected to give a weaker x-ray diffraction pattern. In addition, the extensive interactions between the thick filaments and thin filaments in relaxed cardiac muscle, compared with skeletal muscle, may contribute to the weakness of the relaxed cardiac muscle x-ray diffraction pattern. Because I am examining single thick filaments selected as not having bound actin, this problem would not exist in my studies.

Weisberg and Winegrad (1996, 1998), in recent electron microscope studies on rat cardiac muscle, have also presented evidence that the filaments have a 43-nm helical or near-helical periodicity. In addition, their studies suggested that filaments with the α-myosin heavy chain have a better periodicity than those with the β-myosin heavy chain. Although these studies are in basic agreement with my results, the rat filaments were not as well-ordered and the optical diffraction patterns from them were relatively weak compared with those shown here. Since VanBuren et al. (1995) have reported that the normal adult rabbit cardiac muscle has a composition of ~70% V3 isoform of myosin, it is likely that the majority of the rabbit filaments examined by me consist of myosin with the β-myosin heavy chain. Thus, if similar to the filaments in the rat, the filaments examined here should be less well-ordered than those with the α-myosin heavy chain. It will thus be important to determine whether even more highly ordered rabbit cardiac filaments can be obtained from hearts containing primarily the α-myosin heavy chain.

The diameter of the isolated rabbit cardiac thick filaments was 31.05 $\pm$ 1.55 nm (n = 100), which is similar to the diameter of 30.4 $\pm$ 3 nm (n = 174) which we have previously measured for frog thick filaments and 31.5 $\pm$ 2.1 nm (n = 104) for fish thick filaments negatively stained on carbon (Almaguel et al., 2000). Although Winegrad et al. (2000) have reported differences in diameter of rat cardiac filaments depending upon the level of phosphorylation of MyBP-C, no clear evidence of variation in diameter or cross-bridge pattern that might have resulted from variations in the degree of phosphorylation of MyBP-C on the rabbit filaments was observed. The problem of variable flattening of specimens during negative staining (Almaguel et al., 2000) and slight variations in the electron microscope magnification complicates the interpretation of small variations in diameter. In the present experiments the 43-nm axial periodicity of the filaments was used as a ruler to calibrate the magnification of each measured filament, thus increasing the accuracy.

The periodic structure of the isolated rabbit cardiac thick filaments shown here is significant because the filaments are highly ordered enough to allow the Fourier-based com-

puter image analysis that was previously used to analyze the structure of vertebrate skeletal muscle thick filaments (Kensler and Stewart, 1983, 1989; Stewart and Kensler, 1986; Kensler et al., 1994; Kensler and Woodhead, 1995). These previous studies on skeletal muscle thick filaments allowed us to determine the number of cross-bridges at each cross-bridge level (Kensler and Stewart, 1983, 1989; Kensler et al., 1994; Kensler and Woodhead, 1995) and, in the case of frog thick filaments, allowed a full three-dimensional reconstruction of the cross-bridge arrangement to be determined (Stewart and Kensler, 1986). We are currently collecting similar data for the isolated rabbit cardiac thick filaments. These studies should allow both the differences and similarities between cardiac and skeletal muscle thick filaments to be determined, and are critical for interpreting the effect of myosin isotype and phosphorylation of MyBP-C protein on the filament structure.

Interactions of the relaxed cardiac thick filament with actin

Cardiac muscle is known to differ from skeletal muscle in a number of physiological properties, including its high resting tension and steep relationship between active force and sarcomere length (Frank-Starling relation). Evidence suggests that some of these differences relate to the properties of the cardiac myosin cross-bridge and its interactions with actin (Fuchs and Wang, 1997). In support of this idea, the x-ray diffraction results of Matsubara (Matsubara and Millman, 1974; Matsubara et al., 1989) suggest that up to 20% of the cross-bridges in relaxed cardiac muscle may remain associated with actin in a non-force-producing conformation, thus providing an explanation for the higher resting tension in cardiac muscle. The greater tendency shown here for the isolated rabbit cardiac filaments to remain attached to adjacent actin thin filaments under relaxing conditions (as compared with isolated skeletal muscle thick filaments) is consistent with these results of Matsubara (Matsubara and Millman, 1974; Matsubara et al., 1989). This increased interaction between the cardiac thick and thin filaments may also provide an explanation for the greater difficulty in isolating cardiac muscle thick filaments compared with skeletal muscle.

Considering the physiological properties of cardiac muscle, it is significant that cardiac muscle contracts in a continuous rhythmic pattern. As such, there may be advantages to always having a fraction of the myosin cross-bridges associated with the actin thick filament in a non-force-producing conformation and, thus, ready for the next contractile cycle. Abundant evidence now exists for the presence of such weak-binding cross-bridge states in muscle (Brenner et al., 1982, 1984; Xu et al., 1997).

If the attached cross-bridges in my preparations represent weak-binding cross-bridges fixed in place by the uranyl acetate, the images would be of considerable interest. In most previous electron microscope structural studies of weak-binding cross-bridges, isolated myosin heads bound to actin have been examined (Pollard et al., 1985, 1993; Applegate and Flicker, 1987; Frado and Craig, 1992), and it has been reported that the binding is either disordered or similar to the rigor conformation. Applegate and Flicker (1987) and Frado and Craig (1992) examined myosin heads covalently cross-linked to actin and found that, under conditions that promote weak binding, the myosin heads appeared to be disordered. These studies were criticized, however, because they may not have distinguished weak-binding cross-bridges from detached myosin heads tethered only by the cross-linker (Pollard et al., 1993).

Pollard et al. (1993), in contrast, used rapid freezing of untethered myosin heads binding to actin under different nucleotide states. They reported that many of the myosin heads under weak-binding conditions appeared to bind to actin in an angled conformation ($\sim 40^{o}$) similar to that seen in rigor. Unfortunately, the polarity of the actin filaments and thus of the binding was not absolutely determined. Although they assumed that the angle of binding of the myosin heads was uniformly toward the pointed end of the actin, and thus was similar to rigor, they noted that it could have been at 90^{o} to this, and thus backward from rigor. The latter interpretation would be consistent with the results presented here.

In addition to the problems in interpretation, most of these studies have not directly considered the steric constraints that the near-helical arrangement of the cross-bridges on the thick filament surface may place on their preforce attachment to actin. Unlike free myosin heads in solution, the myosin heads in the thick filament are attached to and part of a macromolecular structure that may limit their freedom of motion. The isolated cardiac filament preparations shown here provide an opportunity to study the binding of the cross-bridges to actin under the geometric constraints imposed by their periodic arrangement on the thick filament.

In my preparations these attached cross-bridges often have a relatively ordered nonrigor attachment. Computed Fourier transforms clearly show that the thick filaments retain their highly ordered relaxed structure despite their attachment to the actin, thus making it unlikely that the images represent filaments with their cross-bridges in a rigor conformation. Both the number of thick filaments with bound actin and the number of cross-bridge attachments between the thick and thin filament make it unlikely that they are simply fortuitous contacts between the myosin heads and actin.

Careful inspection of the micrographs suggests that the majority of the cross-bridges attach to the actin in a narrow range of different angles, consistent with a mismatch between the 43-nm periodicity of the thick filament and the 38.7-nm periodicity of the actin target zones while still remaining close to their expected position in the helical

array of the relaxed filament. It is important that, while the angle of attachment often appears similar, it is clearly not identical, and there is a degree of freedom in the attachment angle. This is consistent with the nonstereospecific binding properties expected for "weak-binding" cross-bridge attachment to actin (Xu et al., 1997), and with EPR studies suggesting that weak-binding cross-bridges are disordered (Fajer et al., 1991). At the same time, the relatively ordered attachment points of the heads to the thick filament would explain the persistence of the myosin layer line pattern under conditions thought to represent weak-binding (Xu et al., 1997). The images would also be consistent with the data of Hudson et al. (1997) from fish skeletal muscle that suggest the resting-state myosin heads must swing axially by up to 150 Å to reach rigor.

Recent evidence from Xu et al. (1999) suggests that the $M \cdot ADP \cdot P_i$ state is required for helical order in the thick filaments of skeletal muscle, and that this state may be in rapid equilibrium with other weak-binding states in the ATPase cycle (i.e., $M \cdot ATP$ and $A \cdot M \cdot ADP \cdot P_i$). Other evidence from x-ray crystallography studies has defined at least three structural conformations (states I, II, III) of the myosin head during the cross-bridge cycle (Rayment et al., 1993a,b; Smith and Rayment, 1996; Holmes, 1997; Dominguez et al., 1998). State III (Houdusse et al., 1999), proposed to correspond to the detached ATP state of myosin, has a relatively straight conformation, with the lever arm in the up-position. State II (Dominguez et al., 1998), proposed to correspond to a pre-stroke conformation, has a bent conformation, with the lever arm in the up-position. State I, proposed to correspond to the rigor state (Rayment et al., 1993a,b), has a relatively straight conformation, with the lever arm in the down-position. Tomographic studies of insect flight muscle (IFM) appear consistent with this concept (Reedy, 2000). Many of the cross-bridge attachments shown here in the relaxed cardiac thick filament-thin filament complexes appear relatively straight (Fig. 4 *c*), and point toward the *z*-line (anti-rigor orientation), which should correspond to the lever arm in the up-position. Their appearance is thus most consistent with that expected for state III. However, because the images represent the projection of density into a single plane, the apparent shape and angle of the cross-bridges will depend upon both the axial tilt and azimuthal angle of the myosin heads, and the presence and orientation of the two heads relative to each other; thus the absolute shape and orientation of the myosin heads cannot be ascertained from the single images. Therefore, at present, either a state III or state II conformation of the cross-bridges appears consistent with the data, but a state I (rigor-like) conformation does not. Further work will be necessary to resolve this question.

The isolated rabbit cardiac thick filament preparation described here may thus provide a model system for determining the detailed macromolecular structure of the cardiac thick filament, and also provide a system for studying the interactions of cross-bridges under relaxed conditions with actin. Continued study of these filaments may allow insight into the differences in the contractile mechanisms of cardiac and skeletal muscles.

I thank my colleagues at the University of Puerto Rico School of Medicine for their helpful comments and suggestions.

This work was supported by Grant 9607749S from the American Heart Association through its Puerto Rican Affiliate, Minorities Basic Research Support Grant S06 GM08224 from the National Institutes of Health and, in part, by funding from "Research Centers in Minority Institutions" Award G12RR-03051 from the National Center for Research Resources, National Institutes of Health.

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