Arrangement of Myosin Heads on *Limulus* Thick Filaments

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Abstract. The two myosin heads with a single surface subunit on thick filaments from chelicerate arthropod muscle may originate from the same, or from axially sequential molecules, as suggested by three-dimensional reconstructions. The resolution attained in the reconstructions, however, does not permit one to distinguish unequivocally between these two possible arrangements. We examined the effect of 0.6 M KCl on relaxed thick filaments separated from *Limulus* muscle and filaments in which nearest myosin heads were cross-linked by the bifunctional agent, 3,3′-dithiobis[3′(2′)-*O*-[6-propionylamino)hexanoyl]adenosine 5′-triphosphate ($bis_{22}ATP$), in the presence of vanadate (V_i). In high salt, surface myosin dissolved from both native, relaxed filaments and those exposed to 1–2 mM dithiothreitol after cross-linking, but was retained on filaments with cross-linked heads. Since $bis_{22}ATP$ must form intermolecular bonds between myosin heads within each subunit to prevent myosin solubilization in high salt, we conclude that each of these heads originates from a different myosin molecule, as was previously predicted by the reconstructions.

THREE-dimensional reconstructions of images of relaxed *Limulus*, scorpion, and tarantula thick filaments reveal the four-stranded, helical array of subunits (crossbridges) on their surfaces (Stewart et al., 1981, 1985; Crowther et al., 1985), and also suggest that the two myosin heads within a subunit are antiparallel in orientation and arise from axially sequential myosin molecules (Stewart et al., 1985; Crowther et al., 1985) (Fig. 1). This arrangement differs from common models of thick filament structure, in which each crossbridge is composed of both heads of the same myosin monomer, oriented in parallel (Harford and Squire, 1986). The resolution attained by the reconstructions, however, is insufficient to permit unambiguous determination of the arrangement of individual myosin heads within the surface subunits of relaxed filaments.

To decide whether the myosin heads within each subunit originate from the same or from different myosin molecules, we used the bifunctional agent, 3,3′dithiobis[3′(2′)-*O*-[6-(propionylamino)hexanoyl]adenosine 5′-triphosphate($bis_{22}ATP$),[1] courtesy of Dr. Ralph Yount, Washington State University, Pullman, WA), which has a 2.8 nm, disulfide bond-containing region linking the ribose oxygens of two ~1 nm ATP molecules (Munson et al., 1985), to cross-link nearest-neighbor myosin heads on filaments isolated from *Limulus* telson muscle. Excess vanadate ion (V_i) was added to $bis_{22}ATP$ to form a bound nucleotide inhibitor, $bis_{22}ATP \cdot V_i$, and to maximize cross-linking (Munson et al., 1985). We examined the effect of 0.6 M KCl on the structure of untreated (relaxed) and treated (heads cross-linked) filaments. If both heads within each subunit originate from the same myosin molecule (Fig. 1 *B*), the cross-linker should form intramolecular bonds between them. High salt, then, should dissolve the surface myosin, leaving only paramyosin cores, as occurs with untreated filaments (Levine et al., 1982). If each of the two heads arises from a different myosin molecule (Fig. 1 *A*), $bis_{22}ATP \cdot V_i$ may form intermolecular bonds between them. In this case, high salt might release the surface myosin as long, helical strands, or, with extensively cross-linked surfaces, the filaments might remain intact.

Materials and Methods

Filament Preparation

Thick filaments were separated from bundles of *Limulus* telson muscle in relaxing solution: 0.1 M KCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, 0.4 mM NaN_3, 7 mM phosphate buffer (or 10 mM imidazole buffer), pH 6.8, as previously described (Kensler and Levine, 1982*a*, *b*). Filament suspensions were placed on carbon films (either very thin carbon, 5–7 nm thick, over holes in a formvar supporting film, or medium carbon, 10–15 nm thick, unsupported by formvar) on electron microscope grids. Excess fluid was drawn off and the filaments adsorbed to the carbon were rinsed with relaxing solution. Some grids were negatively stained with 1% aqueous uranyl acetate and others were unidirectionally shadowed with platinum-carbon (Kensler and Levine, 1982*a*, *b*) without additional incubation in other solutions. These were immediately examined in the electron microscope to ensure that the filaments retained well-ordered, "relaxed" surface arrays of myosin.

Incubation of Filament Samples on Experimental and Control Media

We performed all incubations by inverting the grids onto which the filaments were adsorbed onto the surfaces of drops of the different solutions. For incubations of longer than 5 min, we transferred the grids to new drops of solution at 5-min intervals. Some sample grids were rinsed with the last solution

1. *Abbreviations used in this paper*: $bis_{22}ATP$, 3,3′-dithiobis[3′(2′)-*O*-[6-(propionylamino)hexanoyl]adenosine 5′triphosphate; IAA, iodoacetic acid; ll, layer line(s); V_i, vanadate ion.

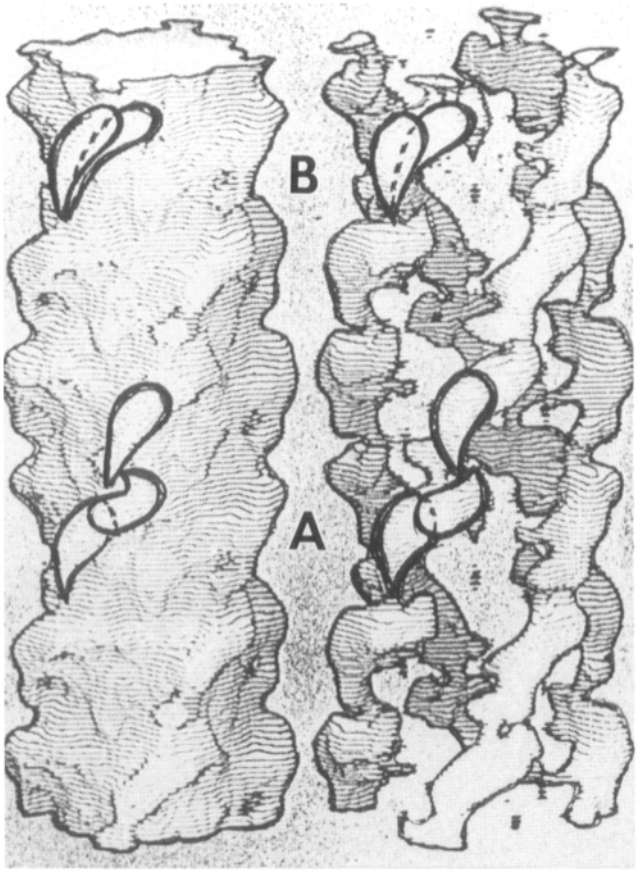

Figure 1. Computer models of three-dimensional reconstructions of *Limulus* thick filaments produced by Fourier-Bessel inversion (Stewart et al., 1985). Equatorial data were preserved in the left image but omitted from the right one. The filaments are tilted toward the viewer and the bare zone is toward the bottom of the figure. The bilobed appearance of the surface subunits that comprise the helical array of crossbridges is seen on both models. The drawn myosin heads at *A* illustrate the orientation of myosin heads if each head within a subunit originates from a different, axially sequential, myosin molecule. The orientation of the myosin heads drawn at *B* is that to be expected if each head within a subunit originates from the same myosin molecule. (This illustration was provided by Dr. M. Stewart, MRC Laboratory, Cambridge, UK.)

on which they had been incubated (except those incubated last on $bis_{22}ATP$ or $bis_{22}ATP{\cdot}V_i$) and either negatively stained or unidirectionally shadowed with platinum-carbon for examination in the electron microscope. We performed all incubations (including that just in relaxing solution) both in the presence and the absence of a 10-fold molar excess of freshly prepared iodoacetic acid (IAA) over myosin in the preparation. We also examined the appearance of untreated filaments in relaxing solution to which excess vanadate ion (V_i) had been added, again, in the presence and absence of IAA.

Experimental Incubations

Step 1. 15 min on rigor buffer: 0.1 M KCl, 2.5 mM $MgCl_2$, 0.4 mM NaN_3, 10 mM imidazole buffer, pH 6.8. This incubation removes bound ATP from the myosin heads of the thick filaments and is necessary since

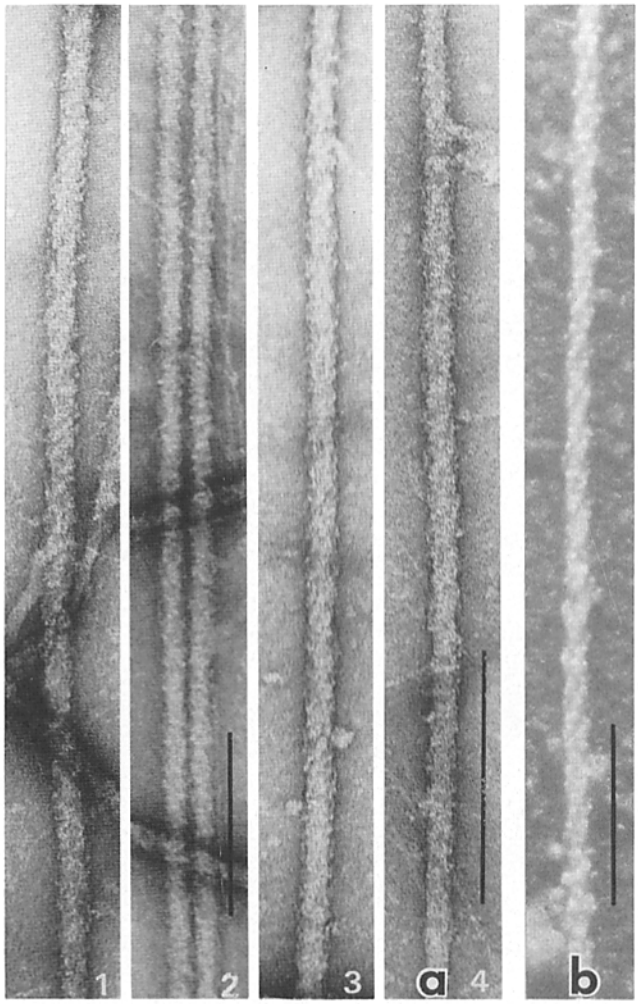

Figure 2. Electron micrographs of (*a1–4*) negatively stained and (*b*) unidirectionally shadowed filaments and (*c*) an optical transform obtained from a negatively stained specimen. Bare zones are toward the bottom of each filament. Filaments in *a1* and *a2* were rinsed with relaxing solution without IAA before staining. Filaments in *a3* and *a4* were rinsed with relaxing solution containing a 10-fold molar excess of IAA over myosin before staining. 1 mM sodium vanadate was present in the relaxing solution used to rinse filaments in *a2* and *a4*, but not those in *a1* and *a3*. Neither IAA or vanadate was present in the relaxing solution used to rinse the filament seen in *b*. The transform in *c* was obtained from a filament treated with relaxing solution containing vanadate but not IAA. All relaxed filaments, with or without IAA or vanadate, show the typical ordered array of myosin subunits on their surfaces (*a* and *b*), clearly visible as bumps along helical paths in *b*, and produce the expected (Kensler and Levine, 1982*a*) transform (*c*). (*a*) Filaments *1*, *3*, and *4*, ×164,000. Bar, 0.2 μm. (*a*) Filaments in *2* and (*b*), ×121,000. Bar, 0.2 μm.

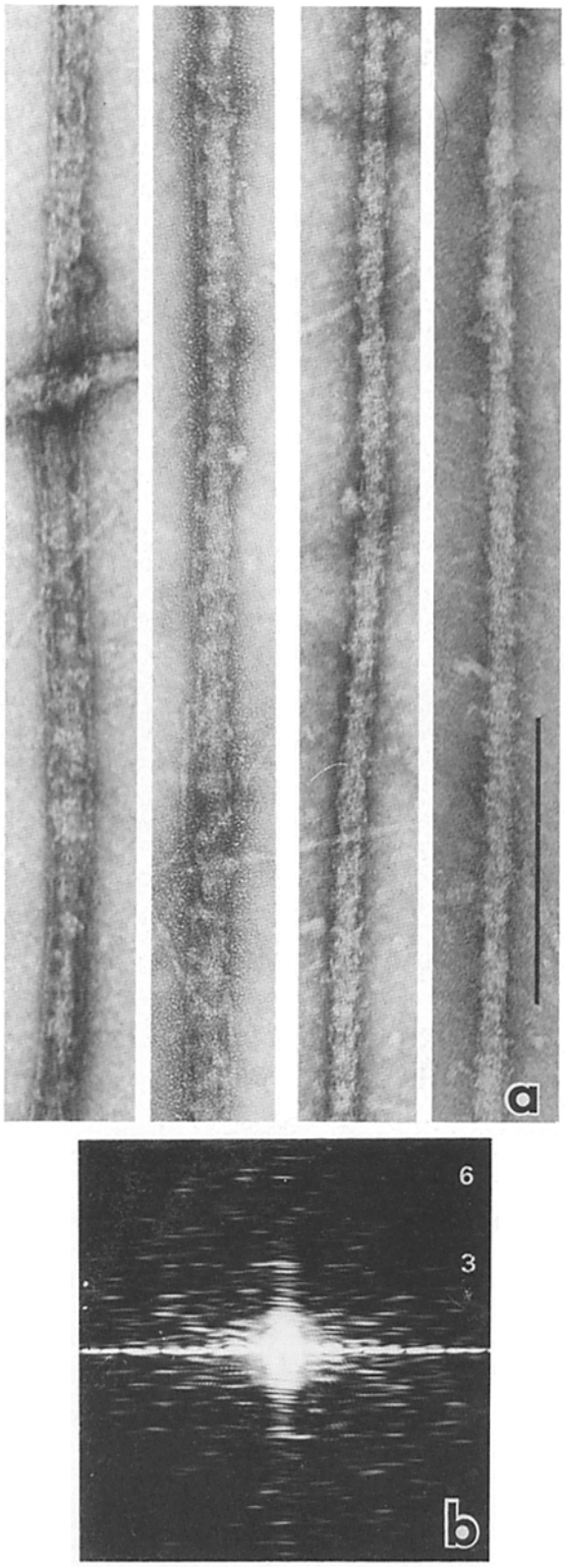

Figure 3. (*a*) Electron micrographs of negatively stained thick filaments that were incubated for 15 min on rigor buffer and (*b*) optical transform obtained from one such filament. Bare zones are toward the bottom of each filament. Note the loss of helical order from the surface of the filaments and the outward position of the myosin heads that bind to actins on neighboring thin filaments in the two left-hand micrographs. (*a*) ×186,000. Bar, 0.2 μm. There is little indication of helical order in the transform in *b*. A strong meridional reflection is seen on the third ll and a weak one on the sixth ll. There may be some reflections associated with the first ll present, but the fourth ll is absent.

$bis_{22}ATP$ has a lower affinity for myosin than does MgATP (Munson et al., 1985).

Step 2. 2–5 min on $bis_{22}ATP$/rigor buffer (above) plus 1 mM $bis_{22}ATP$. This incubation saturates the myosin heads with the cross-linker before stabilization of the cross-links in the succeeding step.

Step 3. 15 min on $bis_{22}ATP \cdot V_i$/rigor buffer (above plus 1 mM $bis_{22}ATP$ and 2 mM sodium vanadate. This incubation stabilizes the cross-links between nearest-neighbor myosin heads.

Step 4. 15 min on 0.6 M KCl. This incubation tests for the solubility of the myosin when nearest-neighbor heads are cross-linked.

Control Incubations

Control 1: No Cross-links Formed. 15-min incubation of relaxed thick filaments, never exposed to any of the steps in the experimental procedure, on 0.6 M KCl. This incubation tests the solubility of myosin on untreated, relaxed thick filaments.

Control 2: Cross-links Severed.

STEP 1. 15-min incubation of filaments with cross-linked heads on rigor buffer plus 2 mM dithiothreitol (DTT), a sulfhydryl reagent that reduces the disulfide bond within the cross-linker.

STEP 2. 15-min incubation of filaments with cross-linked heads, subsequently exposed to rigor buffer containing 2 mM DTT, on 0.6 M KCl. This incubation tests the solubility of the myosin on filaments that have had cross-links between nearest-neighbor myosin heads severed.

Electron Microscopy

Electron microscopy of all negatively stained and unidirectionally shadowed specimens was performed in a JEOL 100CX electron microscope at an accelerating voltage of 80 kV, with an anticontamination device operating. Micrographs of filaments were obtained at initial magnifications of 19,000, 36,000, and 48,000, on Kodak EM film.

Image Analysis

Where sufficient order was apparent on the images of thick filaments, we obtained optical transforms from them, as previously described (Kensler and Levine, 1982*a*).

Results

Samples of *Limulus* thick filaments prepared in relaxing solution and either negatively stained or unidirectionally shadowed (Kensler and Levine, 1982*a*, *b*) retained well-ordered surface arrays of crossbridges (Fig. 2, *a* and *b*) and produced optical transforms typically associated with the relaxed state (Fig. 2 *c*). As was the case for all experimental and control samples examined, the filaments appeared the same whether or not a 10-fold molar excess of IAA over myosin was present in the incubation or rinsing solutions (compare filaments in Fig. 2 *a*: filaments *1* and *2*, no IAA; filaments *3* and *4*, with IAA). Addition of excess sodium vanadate to the relaxing solution had no effect on the surface array of myosin on relaxed thick filaments (Fig. 2 *a*: filaments *1* and *3*, no vanadate; filaments *2* and *4*, with vanadate).

After 15 min on rigor buffer to remove bound ATP (experimental step 1), filaments became disordered (Fig. 3), as previously reported (Levine et al., 1986). Myosin heads extended away from the surface of rigor filaments and bound to thin filaments if the latter were in proximity to the thick

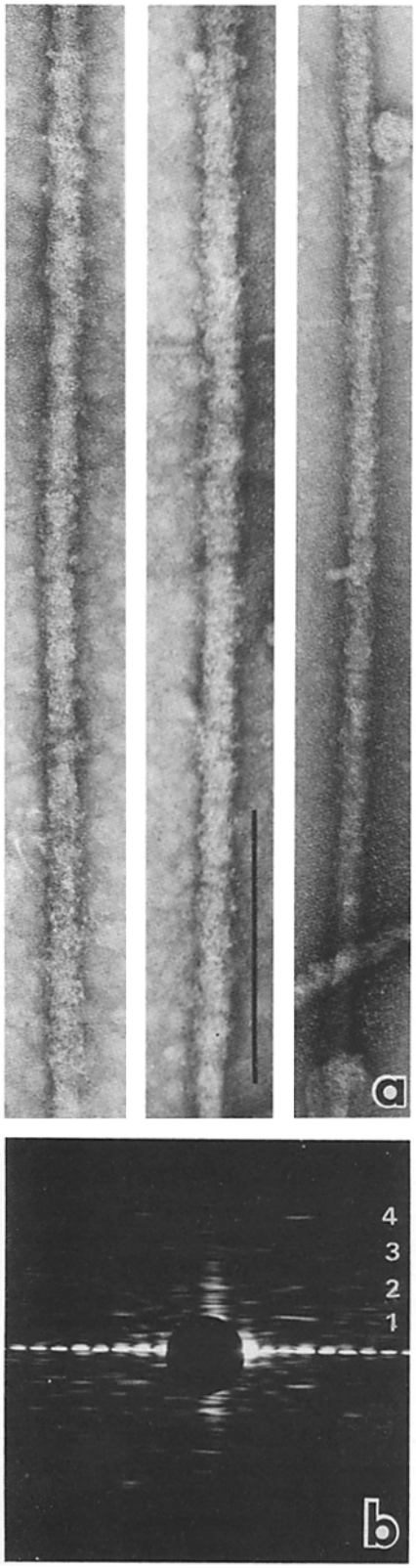

Figure 4. (*a*) Electron micrographs of thick filaments that were first incubated for 15 min in rigor buffer, then for 5 min on $bis_{22}ATP$ without vanadate, before negative staining and (*b*) an optical transform obtained from one such filament. Bare zones are toward the bottom of each filament. Note the evidence of a 43.8-nm repeat periodicity, especially in the right-hand filament in *a*. This is supported by the return of reflections on the first and fourth ll, seen in the transform in *b*. The appearance of filaments that have myosin heads cross-linked with $bis_{22}ATP$ and stabilized with vanadate is the same as that of these filaments; furthermore, incubation of filaments with cross-linked, stabilized myosin heads on rigor buffer does not alter their appearance. (*a*) ×186,000. Bar, 0.2 μm.

(Fig. 3 *a*) and little order remained in the optical transform (only the third and sixth meridional reflections and a hint of the first layer line [ll]; see Fig. 3 *b*). This appearance indicated the removal of MgATP (most likely $MgADP \cdot P_i$) from the ATP-binding site on the myosin heads.

The myosin heads moved back to a position close to the filaments' shafts after 5 min on $bis_{22}ATP$ (experimental step 2), indicating binding of the cross-linker to myosin (Fig. 4, *a* and *b*). In some instances the relaxed pattern reappeared, showing a return to helical order (Fig. 4 *b*). After 15 min of additional incubation on $bis_{22}ATP$ plus excess V_i to stabilize the cross-links (experimental step 3), the myosin heads remained close to the filaments' shafts. There was essentially no change from the appearance of the filaments in $bis_{22}ATP$ without vanadate (Fig. 4). 15 min of additional incubation on rigor buffer (without DTT) did not affect filament structure further, indicating that the cross-linker was not removed by incubation in ATP-free solution (rigor buffer). When 1–2 mM DTT was added to the rigor buffer, however, myosin heads extended away from the filaments' shafts in the "rigor" position (Fig. 5). This result indicated that the disulfide bond linking the two halves of the cross-linker had been severed by the reducing agent, DTT, allowing the outward movement of myosin heads to occur in rigor buffer, as previously observed on filaments with non-cross-linked heads (compare with Fig. 3).

Although filaments still appeared intact after 15 min on 0.6 M KCl subsequent to cross-linking with $bis_{22}ATP \cdot V_i$, distortion of the cross-bridge lattice was frequently seen (Fig. 6). This is shown clearly in the shadowed specimen, Fig. 6 *b*, which displays a bumpy surface, indicative of the retention of myosin, but lacks the helical order seen in the untreated, relaxed filament (Fig. 2 *b*). Many filaments, nevertheless, did retain a fair degree of helical order, which is illustrated by several of the negatively stained filaments in Fig. 6 (*a* and *d*), and further, by the presence of some of the layer lines associated with the helical order of relaxed filaments in the optical transforms (Fig. 6, *c* and *e*). Thus, the cross-linked surface myosin molecules were not removed from the filaments' surfaces even after a lengthy exposure to high salt. In control experiments, however, similar exposure to high salt caused removal of surface myosin from relaxed filaments not incubated with cross-linker (control experiment 1) (Fig. 7, *a* and *b*), as well as from those incubated on rigor buffer containing 2 mM DTT, after cross-linking (control experiment 2) (Fig. 8). In both cases, only smooth paramyosin cores remained; occasionally a few, widely spaced myosin molecules "dangled" from them (Fig. 8). It should be noted that after such a long exposure to high salt, even the paramyosin cores of control filaments started to disaggregate: variability in the diameters of the cores is illustrated in Figs. 7 and 8.

A total of eight filament preparations were subjected to the experimental and control incubations and all gave identical results. In addition, in the case of four of these preparations, parallel incubations were run in the presence of a 10-fold mo-

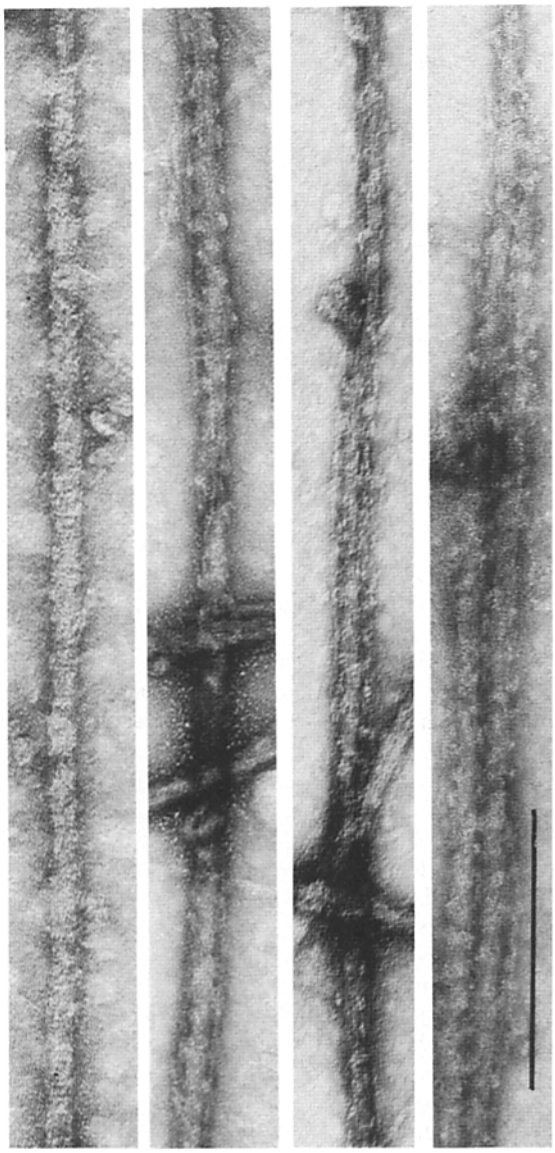

Figure 5. Electron micrographs of thick filaments that had myosin heads cross-linked and stabilized by incubation on $bis_{22}ATP \cdot V_i$, then were incubated on rigor buffer containing 2 mM DTT for 15 min. Bare zones are toward the bottom of each filament. Note the return to the rigor appearance of the filaments: the myosin heads extend away from the filaments' surfaces and bind to available thin filaments. Order is lost. This indicates that the disulfide bond of the cross-linker was reduced (and severed) by DTT, allowing the effect of ATP depletion to become evident. ×186,000. Bar, 0.2 μm.

lar excess of IAA over myosin (assuming the myosin content of the filament preparation to be 0.15 mg/ml), in order to mask the sulfhydryl groups on myosin and thus prevent the possibility of sulfhydryl–disulfide exchange between these and $bis_{22}ATP$. Thus, the formation of cross-links between a myosin head to which one-half of a cross-linker is bound and another site on the myosin molecule, including the reactive sulfhydryl group on each myosin head, was prevented. The results of incubations in the presence of IAA were identical to those in its absence (some filaments from IAA incubations are illustrated in Figs. 2 *a* (filaments *3* and *4*) and 6, *d* and *e*, indicating that during the procedure, no spurious binding of cross-linker to non–ATP-binding, sulfhydryl-containing sites on myosin occurs.

Discussion

These results indicate that $bis_{22}ATP \cdot V_i$ effectively cross-links myosin heads on *Limulus* thick filaments. Since the results of incubations in IAA-containing media were identical to those in media without IAA, the possibility that sulfhydryl–disulfide exchange occurs between the cross-linker and sulfhydryl groups anywhere on myosin can be eliminated. Furthermore, we are assured that at the pH (6.8) at which all of the incubations are performed, even the most reactive thiol group on myosin is essentially stable and does not participate in such exchange (Yount, R., personal communication).

Whether or not excess sodium vanadate is present, the appearance of relaxed thick filaments is the same. Thus any structural effect of the procedures on the surface array of myosin heads is not due to the presence of V_i in the stabilized, cross-linked structure.

Since the surface subunits return to occupy positions close to the filaments' shafts, and may even display an array similar, if not identical to the relaxed pattern, in $bis_{22}ATP$, after the disordering effects of ATP depletion, the cross-links most likely occur between heads within each subunit: heads that are closest to each other in the relaxed state (Stewart et al., 1985). We suggest that during the incubation on $bis_{22}ATP$, before the addition of vanadate, one cross-linker may bind to each of the ATP-binding sites available (one per myosin head). Since we have previously shown that reincubation of rigor *Limulus* thick filaments in relaxing solution produces a return to the relaxed crossbridge array (Levine et al., 1986), a similar effect may occur in the present situation.

Retention of all of the myosin heads on filaments treated with cross-linker, even after 15 min on 0.6 M KCl, implies that the linkages formed between the myosin heads are intermolecular in nature. Sets of such linkages, following the helical paths of the surface strands of myosin, may be responsible for stabilizing filament structure and producing the resistance of myosin to solubilization at high ionic strength. Incubation of filaments with cross-linked myosin heads in rigor buffer containing the sulfhydryl reagent, DTT, breaks the disulfide bond of $bis_{22}ATP$ and, as seen in Fig. 8, myosin dissolves off of the filaments in high salt, just as it does when untreated, relaxed filaments are exposed to 0.6 M KCl. This further supports the notion that the cross-linked heads originate from different myosin molecules.

The distortion of the helical crossbridge array that is frequently seen on filaments first treated with $bis_{22}ATP \cdot V_i$ and then exposed to high salt may be due to one or more of several factors. First, one must consider the constraint that the cross-linker itself imposes on the geometry of the two myosin heads within each subunit. As seen in Fig. 9 *b*, the drawn triangle shows the distances between axially sequential origins of myosin heads and defines the distance spanned by heads originating from such different sites in comprising one surface subunit. The axial displacement of the sites of origin of heads between successive crossbridge levels on *Limulus* thick filaments is 14.5 nm (*side a* in Fig. 9 *b*). The rotational distance, around the filament shaft, between crossbridge ori-

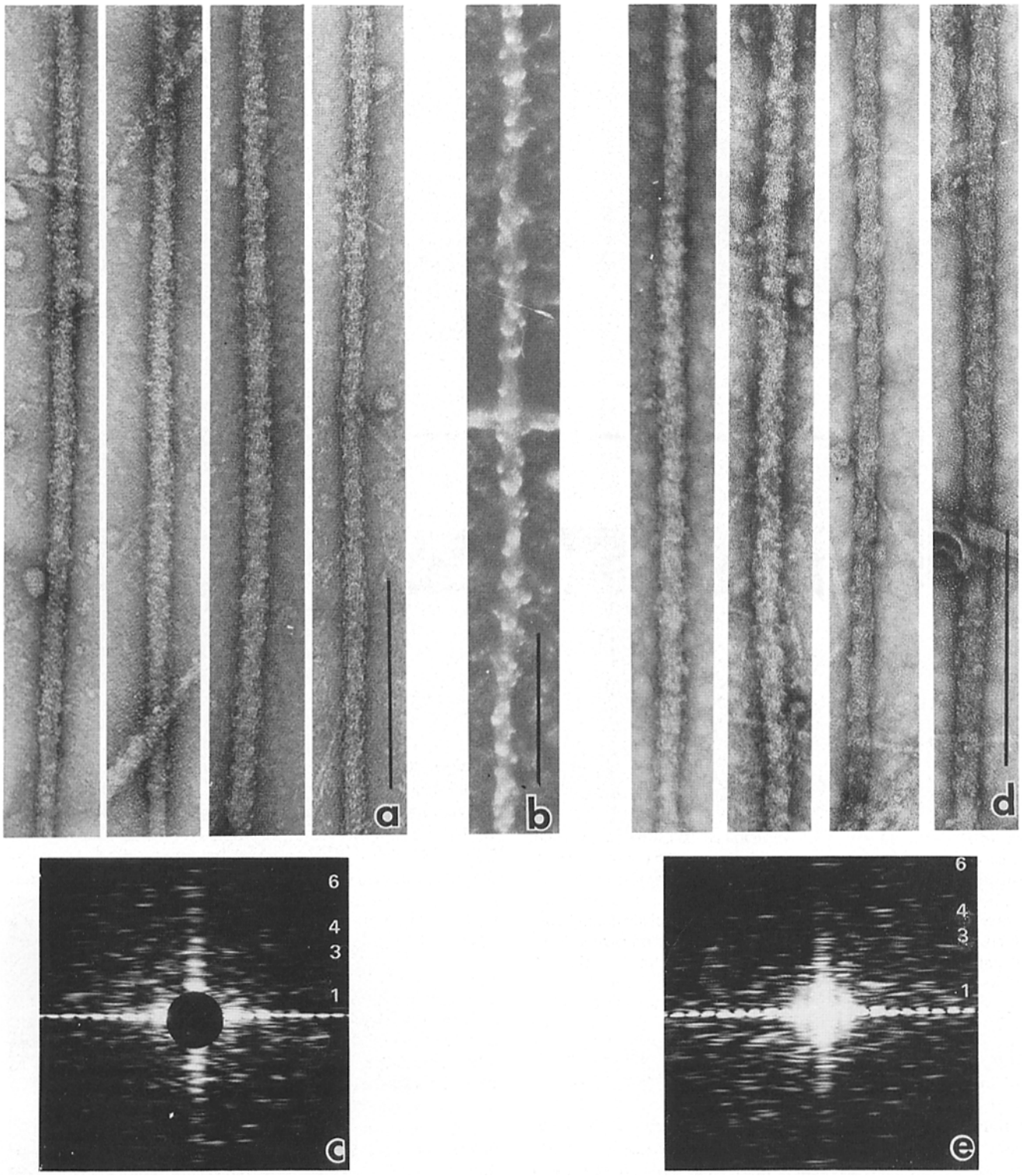

Figure 6. Electron micrographs of filaments that had myosin heads cross-linked and stabilized with $bis_{22}ATP \cdot V_i$, then were incubated for 15 min on 0.6 M KCl before either (*a* and *d*) negative staining or (*b*), unidirectional shadowing with platinum-carbon, and (*c* and *e*) optical transforms obtained from such filaments. Bare zones are toward the bottom of each filament. Filaments in *a* and *b* (and the one from which the optical transform, *c*, was obtained) lacked IAA in the incubation media. Filaments in *d*, and the one from which the optical transform, *e*, was obtained had IAA included in all the incubation media. Note the retention of myosin all along the filament surfaces. There is great variability in the appearance of the surface crossbridge array: order is present in some images but lacking in others, which is especially apparent on the image of the shadowed filament; myosin is present, but the array is clearly distorted. Optical transforms obtained from such filaments show the presence of ll associated with the relaxed state, but there is a lot of background and the patterns are not exceptionally good. (*a* and *d*) ×175,000. Bar, 0.2 μm. (*b*) ×130,000. Bar, 0.2 μm.

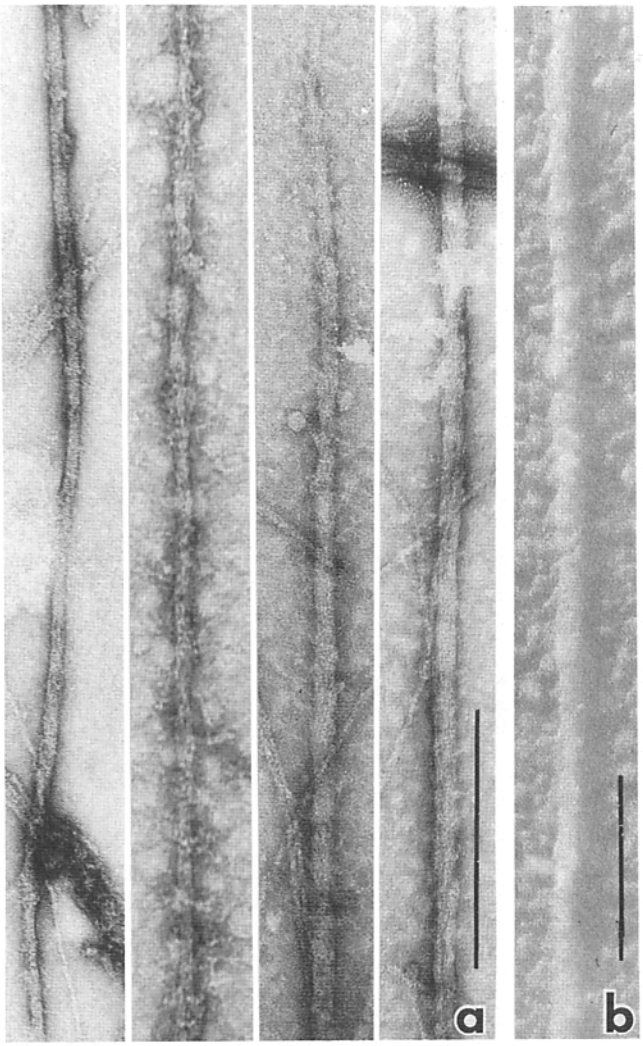

Figure 7. Electron micrographs of filaments that were isolated and rinsed with relaxing solution then incubated for 15 min on 0.6 M KCl, before (*a*) negative staining or (*b*) unidirectional shadowing with platinum-carbon. Note the smooth appearance of these cores. There is variability in their diameters, a common observation, most likely related to the release of paramyosin into solution after such a long incubation in high salt. (*a*) ×169,000. Bar, 0.2 μm. (*b*) ×120,000. Bar, 0.2 μm.

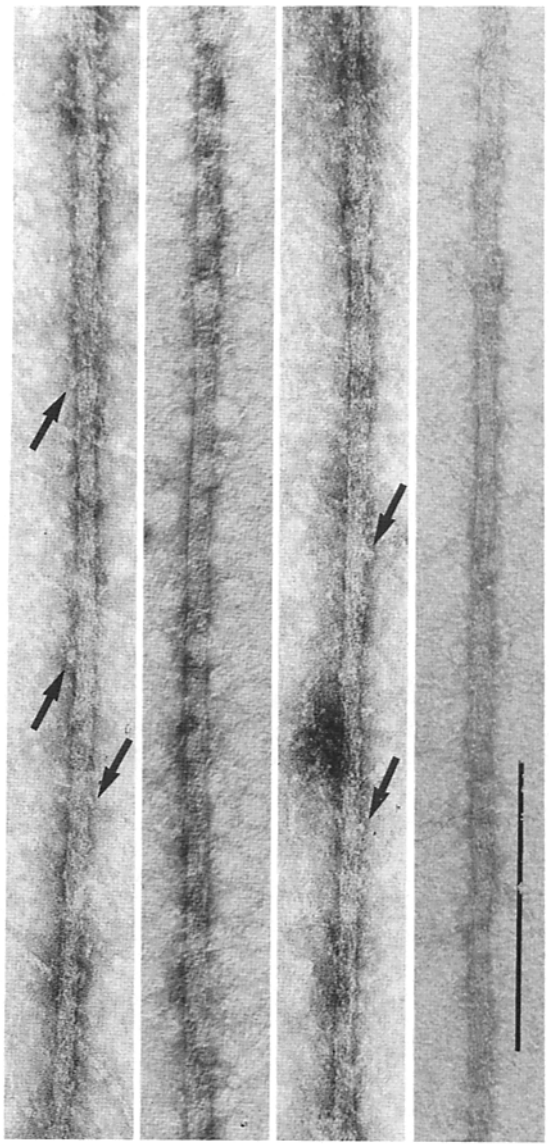

Figure 8. Electron micrographs of filaments that had myosin heads cross-linked with $bis_{22}ATP \cdot V_i$, then were incubated for 15 min on rigor buffer containing 2 mM DTT, and finally were incubated for 15 min on 0.6 M KCl, before negative staining. Note again the loss of surface myosin, indicating that, after the disulfide bond within the cross-linker was reduced and severed in DTT, the myosins were no longer bound to each other along their helical paths and dissolved in high salt. Some of the few remaining myosin molecules are indicated by arrows. ×186,000. Bar, 0.2 μm.

gins on successive levels for these filaments with threefold screw symmetry and fourfold rotational symmetry (Stewart et al., 1981; Kensler and Levine, 1982*a*) is 1/12 the circumference of the filament shaft (diameter 23 nm at the bare zone: Kensler and Levine, 1982*a*; circumference 72.3 nm), or 6.02 nm (*side b* in Fig. 9 *b*). Thus, two myosin heads, each ~16 nm long (Stewart et al., 1985), originating from molecules at axially sequential crossbridge levels, can overlap completely in the length available (hypotenuse of right triangle 15.7 nm; *side c* in Fig. 9 *b*). This estimate takes into consideration the natural curvature of the heads (Stewart et al., 1985; Crowther et al., 1985; Winkelmann et al., 1985). Recently, Tokunaga et al. (1987) have determined that the active site on rabbit myosin lies ~5 nm from the tip of the head. Assuming a similar situation on *Limulus* myosin, the ATP-binding sites of two completely overlapped, antiparallel heads, spanning 16 nm are ~6 nm apart. For the 4.8-nm-long $bis_{22}ATP$ to bridge the distance between the two active sites, one or both of the heads may bend, thus disturbing the relaxed crossbridge pattern. It is possible that there may be as much as a 0.6-nm discrepancy in the position of the active site determined by Tokunaga et al. (1987). In this case, bending of the heads on binding both functional ends of $bis_{22}ATP$ would not be necessary, and the observed distortion might arise from other causes (such as the effect of high salt on filament cores; see below).

The model illustrated in Fig. 9 *c* agrees with our earlier findings on native, relaxed *Limulus* thick filaments (Stewart

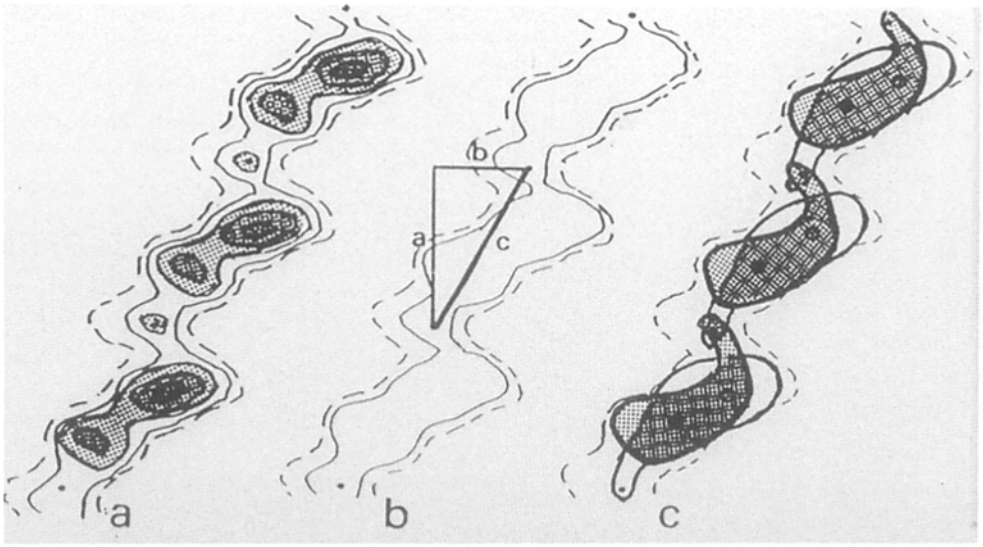

Figure 9. Diagrammatic representation of the subunits on the surface of *Limulus* thick filaments, redrawn from a computed circumferential section (equatorial data omitted) at a radius of 14.5 nm. A quarter turn of three of the four myosin helices is shown. The bare zone is toward the bottom of the figure. (*a*) The density distribution within the subunits is indicated by different levels of shading. (*b*) The drawn triangle demonstrates the distances between axially sequential origins of myosin heads and defines the distance spanned by heads originating from such different sites in comprising a single surface subunit. The axial displacement of the sites of origin of heads between successive crowns is 14.5 nm (*side a*). The rotational distance around the filament shaft between crossbridge origins on successive levels is 6.02 nm (*side b*). The hypotenuse of the right triangle, or the shortest distance that can be traversed between crossbridge levels by the two antiparallel heads within each subunit is 15.7 nm (*side c*). This length is well within most estimates for the length of a myosin head, and is, in fact, very close to the length of that from *Limulus* myosin (16 nm in Stewart et al., 1985). (*c*) A possible structure for the antiparallel arrangement of myosin heads within subunits in one-quarter of one helical strand along the filament surface. Each head spans the distance between axially sequential crossbridge origins. The greatest mass of each head lies distal to its origin. The overlapping heads (*shaded*) point toward the bare zone. The active sites on both heads (*dots*) in each subunit are within range for cross-linking with $bis_{22}ATP$.

et al., 1985) in that: (*a*) the greatest mass of each head lies distal to its origin; (*b*) the overlapping (outer) heads (shaded) point toward the bare zone; (*c*) the density due to the mass of the interior (underlying) head appears first at a low radius and a position distal to the bare zone, within each subunit; and (*d*) the density due to the mass of the exterior (overlapping) head appears at a higher radius and a position proximal to the bare zone. Also, the active sites (dots) on both heads are within range for cross-linking by $bis_{22}ATP$, with minimal distortion.

A second factor to consider is whether or not the cross-linked heads are in the "true" relaxed conformation. Vanadate inhibits myosin ATPase activity by stabilizing the myosin–nucleotide complex either immediately before or just after P_i release (Goodno, 1979; Wells and Bagshaw, 1984). Cross-linked filaments may exhibit a myosin–nucleotide intermediate state different from that associated with relaxation. A third consideration is that the results of structural analyses of both arthropod (Stewart et al., 1981; 1985; Crowther et al., 1985; Kensler and Levine, 1982) and vertebrate (Kensler and Stewart, 1983; 1986; Stewart and Kensler, 1986) thick filaments differ from those of spectroscopic (Thomas, 1987) studies, regarding the ordered appearance of relaxed myosin heads. Spin-label studies describe rigor as the only ordered state of myosin on thick filaments in glycerinated rabbit (Thomas and Cooke, 1980) and insect flight (Thomas et al., 1983) muscle (these are the only muscles that have been found amenable to analysis by electron spin resonance, thus far). Possibly, the highly ordered relaxed crossbridge conformation described by structural analyses is but one, albeit the lowest-energy, state of myosin·MgADP·P_i on thick filaments. Independent movement of individual heads within a subunit (as suggested by electron spin resonance) may bring their active sites close enough to bind the two functional ends of a single $bis_{22}ATP$; V_i then stabilizes them in a position that produces a less ordered surface array than present on naturally relaxed thick filaments. Finally, we would like to point out that the distortion of the crossbridge lattice on the surfaces of filaments with cross-linked myosin is often seen to be greater after exposure to high salt than with cross-linker alone or cross-linker plus vanadate. Since the solubility properties of myosin are associated with the light meromyosin portion of the molecule, the greater disorder displayed by filaments incubated on 0.6 M KCl after having had their myosin heads cross-linked may result from some degree of light meromyosin unpacking from the filaments' shafts, as the myosin responds to the high-salt environment by attempting to disaggregate, rather than from binding the cross-linker. Nevertheless, the fact that the myosin remains on the filaments' surfaces, even after 15 min on 0.6 M KCl, indicates the retention of the cross-link between the heads and strongly supports the separate origin of the two myosin heads within each surface subunit on *Limulus* thick filaments.

Our results may have a bearing on the nature of cooperativity among myosins within a single thick filament and on the involvement of individual myosin heads in the crossbridge cycle. The basis for intermolecular thick filament cooperativity, as seen, for example, during calcium activation of scallop myosin (Chantler et al., 1981) or fibers (Simmons and Szent-Gyorgyi, 1984), may reside in head–head interactions among the myosins comprising each helical strand along the filament. Thus, calcium binding to relatively few myosins within a filament may cause all molecules within a

helical strand to switch on in a cooperative manner. A similar mechanism could be envisioned for filaments where activity is controlled or affected by myosin light-chain phosphorylation, such as those of *Limulus* (Sellers, 1981), tarantula (Craig et al., 1987), or vertebrate smooth (Watanabe, 1985) muscles, if this type of cooperativity is found in these tissues.

The antiparallel arrangement of the two heads within a subunit, if also the case for thick filaments of vertebrate muscle, complicates interpretations of crossbridge movement during the contractile cycle. Future work will need to determine whether a functional crossbridge consists of the two heads of different origin and orientation, or if each head rejoins its intramolecular partner during contractile activity, or, if each head, starting from a different position, goes through the crossbridge cycle independently. Any of these possibilities can be responsible for the loss of helical order and the increased intensity of the 14.3 nm^{-1} meridional reflection, seen during active contraction (Huxley and Kress, 1985). Finally, the disorder observed in spectroscopic analyses of relaxed crossbridges may be explained, in part, by the rotation of oppositely oriented myosin heads around their necks, while maintaining their azimuthal and axial positions. Studies of the effect of 0.6 M KCl on vertebrate and scallop striated muscle thick filaments that have had their myosin heads cross-linked with $bis_{22}ATP \cdot V_i$ are underway; the results of these should resolve some of the questions we have raised.

We thank Drs. Ralph Yount and Andrew Somlyo for their donations of $bis_{22}ATP$ and sodium vanadate, respectively, and acknowledge the photographic assistance of Ms. Harriet King. We appreciate helpful discussions with Drs. Roger Craig, Raul Padron, and John Trinick.

This work was supported by U.S. Public Health Service grants AR-33302 and HL-15835 to the Pennsylvania Muscle Institute (Dr. Levine), AR-30442 (Dr. Kensler), and National Science Foundation grant DCB 8602246 (Dr. Chantler). Dr. Chantler is an Established Investigator of the American Heart Association.

Received for publication 20 June 1988, and in revised form 26 July 1988.

References

Chantler, P. D., J. R. Sellers, and A. G. Szent-Gyorgyi. 1981. Cooperativity in scallop myosin. *Biochemistry.* 20:210–216.

Craig, R., R. Padron, and J. Kendrick-Jones. 1987. Structural changes accompanying phosphorylation of tarantula muscle myosin filaments. *J. Cell Biol.* 105:1319–1327.

Crowther, R. A., R. Padron, and R. Craig. 1985. Arrangement of the heads of myosin in relaxed thick filaments from tarantula muscle. *J. Mol. Biol.* 184:429–439.

Goodno, C. C. 1979. Inhibition of myosin ATPase by vanadate ion. *Proc. Natl. Acad. Sci. USA.* 76:2620–2624.

Harford, J., and J. Squire. 1986. "Crystalline" myosin cross-bridge array in relaxed bony fish muscle. Low angle X-ray diffraction from plaice fin muscle and its interpretation. *Biophys. J.* 50:145–155.

Huxley, H. E., and M. Kress. 1985. Crossbridge behaviour during muscle contraction. *J. Muscle Res. Cell Motil.* 6:153–161.

Kensler, R. W., and R. J. C. Levine. 1982*a*. An electron microscopic and optical diffraction analysis of the structure of *Limulus* thick filaments. *J. Cell Biol.* 92:443–451.

Kensler, R. W., and R. J. C. Levine. 1982*b*. Determination of the hand of the crossbridge helix of *Limulus* thick filaments. *J. Muscle Res. Cell Motil.* 3:349–361.

Kensler, R. W., and M. Stewart. 1983. Frog skeletal muscle thick filaments are three-stranded. *J. Cell Biol.* 96:1797–1802.

Kensler, R. W., and M. Stewart. 1986. An ultrastructural study of cross-bridge arrangement in the frog thigh muscle thick filament. *Biophys. J.* 49:343–351.

Levine, R. J. C., R. W. Kensler, and P. Levitt. 1986. Crossbridge and backbone structure of invertebrate thick filaments. *Biophys. J.* 49:135–138.

Levine, R. J. C., R. W. Kensler, M. Stewart, and J. C. Haselgrove. 1982. Molecular organization of *Limulus* thick filaments. *In* Basic Biology of Muscles: A Comparative Approach. B. M. Twarog, R. J. C. Levine, and M. M. Dewey, editors. Raven Press, Inc., New York. 37–52.

Munson, K. B., M. J. Smerdon, and R. G. Yount. 1985. Cross-linking of myosin subfragment-1 and heavy meromyosin by use of vanadate and a bis(adenosine 5'-triphosphate) analogue. *Biochemistry.* 25:7640–7650.

Sellers, J. 1981. Phosphorylation-dependent regulation of *Limulus* myosin. *J. Biol. Chem.* 256:9274–9278.

Simmons, R. M., and A. G. Szent-Gyorgyi. 1985. A mechanical study of regulation in the striated adductor muscle of the scallop. *J. Physiol. (Lond.).* 358:47–64.

Stewart, M., and R. W. Kensler. 1986. Arrangement of myosin heads in relaxed thick filaments from frog skeletal muscle. *J. Mol. Biol.* 192:831–851.

Stewart, M., R. W. Kensler, and R. J. C. Levine. 1981. Structure of *Limulus* telson muscle thick filaments. *J. Mol. Biol.* 153:781–790.

Stewart, M., R. W. Kensler, and R. J. C. Levine. 1985. Three-dimensional reconstruction of thick filaments from *Limulus* and scorpion muscle. *J. Cell Biol.* 101:402–411.

Thomas, D. D. 1987. Spectroscopic probes of muscle cross-bridge rotation. *Annu. Rev. Physiol.* 49:691–709.

Thomas, D. D., and R. Cooke. 1980. Orientation of spin-labeled myosin heads in glycerinated muscle fibers. *Biophys. J.* 32:891–906.

Thomas, D. D., R. Cooke, and V. A. Barnett. 1983. Orientation and rotational mobility of spin-labeled myosin heads in insect flight muscle in rigor. *J. Muscle Res. Cell Motil.* 4:367–378.

Tokunaga, M., K. Sutoh, C. Toyoshima, and T. Wakabayashi. 1987. Location of the ATPase site of myosin determined by three-dimensional electron microscopy. *Nature (Lond.).* 329:635–638.

Watanabe, S. 1985. Phosphorylation of chicken gizzard myosin: myosin filament hypothesis of calcium regulation. *Adv. Biophys.* 19:1–20.

Wells, C., and C. R. Bagshaw. 1984. The characterization of vanadate-trapped nucleotide complexes with spin-labeled myosin. *J. Muscle Res. Cell Motil.* 5:97–112.

Winkelmann, D. A., H. Mekeel, and I. Rayment. 1985. Packing analysis of crystalline myosin subfragment-1. Implication for the size and shape of the myosin head. *J. Mol. Biol.* 181:487–501.