Disorder induced in nonoverlap myosin cross-bridges by loss of adenosine triphosphate

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ABSTRACT Adenosine triphosphate–dependent changes in myosin filament structure have been directly observed in whole muscle by electron microscopy of thin sections of rapidly frozen, demembranated frog sartorius specimens. In the presence of ATP the thick filaments show an ordered, helical array of cross-bridges except in the bare zone. In the absence of ATP they show two distinct appearances: in the region of overlap with actin, there is an ordered, rigorlike array of cross-bridges between the thick and thin filaments, whereas in the nonoverlap region (H-zone) the myosin heads move away from the thick filament backbone and lose their helical order. This result suggests that the presence of ATP is necessary for maintenance of the helical array of cross-bridges characteristic of the relaxed state. The primary effect of ATP removal on the myosin heads appears to be to weaken their binding to the thick filament backbone; released heads that are close to an actin filament subsequently form a new actin-based, ordered array.

INTRODUCTION

The molecular mechanism of muscle contraction is not understood, although it is generally thought to involve adenosine triphosphate–dependent structural changes in the myosin heads as they interact transiently with actin during the cross-bridge cycle. The structures of relaxed and rigor muscles have received much study because these two states are thought to be related to two of the steps in the cross-bridge cycle. In relaxed muscle (in which the myosin heads bind ATP) most of the cross-bridges are arranged on the surface of the myosin filament in an approximately helical array, reflecting the symmetry of the filament backbone (e.g., Huxley and Brown, 1967; Wray et al., 1975; Wray, 1979; Kensler and Levine, 1982; McLachlan and Karn, 1982), while some may be in a disordered state (Poulsen and Lowy, 1983). In the rigor state (in which the heads lose their nucleotide), cross-bridges in the zone of overlap with actin lose their myosin-based helical order and instead take up the actin filament symmetry by forming strong attachments to actin (Reedy et al., 1965; Huxley and Brown, 1967; Wray et al., 1978). This is made possible by the presence of hinges in the myosin molecule which allow the heads to "search" for appropriately oriented actin monomers (Huxley, 1969). However, the behavior of cross-bridges in the nonoverlap (H-zone) of the sarcomere of rigor muscles is not yet fully understood (e.g., do such "unattached" cross-bridges retain their relaxed helical order or take up some other arrangement?). X-Ray diffraction data have been interpreted as showing that the cross-bridges of relaxed muscle lose their helical order in the nonoverlap zone when ATP is removed (Haselgrove, 1975; Poulsen and Lowy, 1983; Schlichting and Wray, 1986). Electron microscopy of negatively stained, isolated filaments suggests a similar change (Craig and Padrón, 1982; Vibert and Craig, 1985; Levine et al., 1985, 1986; Clarke et al., 1986; Frado and Craig, 1989). Spin-label data have been interpreted as showing that nonoverlap cross-bridges are disordered in the absence of ATP (Thomas and Cooke, 1980; Barnett and Thomas, 1984), and that this disorder is dynamic on the microsecond time scale (Barnett and Thomas, 1984); however, these techniques do not detect the ordering of cross-bridges known to be present in relaxed muscle (Thomas et al., 1980; Thomas and Cooke, 1980; Reedy and Cooke, 1989).

In this paper we report electron microscopic evidence for ATP-dependent structural changes in the thick filaments of whole vertebrate striated muscle prepared by rapid freezing, a technique which provides near in vivo preservation of structure at the macromolecular level. In longitudinal sections of relaxed, freeze-substituted specimens, the helical ordering of the myosin cross-bridges is preserved, whereas removal of ATP causes cross-bridges unattached to actin to move away from the thick filament backbone and lose their helical order. These results provide direct visual evidence of the ordered cross-brdige array in whole muscle inferred from x-ray diffraction and

from negative staining of isolated filaments; our results also provide direct images of the disordered state inferred from x-ray, negative staining, and spin-label data.

MATERIALS AND METHODS

Frog sartorius muscles were chemically skinned by the method of Padrón and Huxley (1984). Small segments of these muscles, in relaxing solution (75 mM KAc, 5 mM MgAc, 5 mM ATP, 5 mM EGTA, 5 mM NaN_3, 2% PVP-40, 15 mM NaPi, pH 7.0) or rigor solution (relaxing solution without ATP), were rapidly frozen, without cryoprotectants,[1] by contact with a liquid helium-cooled copper block (Heuser et al., 1979), and freeze-substituted in 5% osmium tetroxide dissolved in absolute acetone or methanol, as described by Padrón et al. (1988*b*). Specimens were embedded in Araldite or Polybed and longitudinal sections cut perpendicular to the frozen surface using a diamond knife. Sections were picked up on formvar-coated grids, stained with uranyl acetate and lead citrate, and examined in a JEOL 100CX electron microscope operated at 80 kV. Magnifications were calibrated using negatively stained tropomyosin paracrystals, repeat 39.5 nm (Caspar et al., 1969). Optical diffraction patterns of electron micrographs were obtained on a diffractometer built according to the design of Salmon and DeRosier (1981).

RESULTS

Fig. 1 shows longitudinal sections of quick-frozen, demembranated sartorius muscles in the presence and absence of ATP. In the relaxed muscle (Fig. 1 *a*) the thick filaments show a compact structure in which the cross-bridges appear to lie close to the filament backbone, consistent with x-ray diffraction data from living muscle (Huxley and Brown, 1967; Huxley, 1968; Haselgrove, 1980). The A-bands in such specimens frequently show an array of regularly spaced, obliquely oriented stripes extending through both H-zone and overlap zone (best seen by viewing the micrograph at a small angle along the direction of the stripes; Figs. 1 *a* and 2 *a*). Optical diffraction patterns of such A-bands (Fig. 1 *a*, *inset*) show a meridional reflection at a spacing of 13–14 nm, and a layer line at a spacing about three times that of the meridional reflection (~40 nm), corresponding to the oblique stripes. Comparison of this appearance with x-ray diffraction patterns from intact frog sartorius muscle, which show layer lines indexing on a repeat of 43 nm and a strong meridional reflection at 14.3 nm (Huxley and Brown, 1967; Magid and Reedy, 1980; Padrón and Huxley, 1984), suggests that these oblique stripes represent the helical array of myosin heads on the surface of the thick filaments. The somewhat smaller repeats measured in the case of the optical diffraction patterns suggest that 5–10% shrinkage has occurred during processing for electron microscopy.

When ATP is removed, the appearance of the cross-bridge array on the surface of the thick filaments changes dramatically; there are now two populations of cross-bridges (Figs. 1 *b* and 2 *b*). In the overlap region the array is ordered, showing signs of a fine transverse striation and a weaker, oblique periodicity, shorter than that in the relaxed specimens; optical diffraction patterns show a meridional reflection at a spacing of 13–14 nm and a layer line at ~35 nm (Fig. 1 *b*, *inset*), corresponding (after allowing for shrinkage) to the 14.4- and 37-nm reflections in x-ray diagrams of rigor muscle (Huxley and Brown, 1967; Padrón and Huxley, 1984). In the nonoverlap region (H-zone) of the same thick filaments, the cross-bridges have lost their helical order and appear to clump together and to project much further from the filament backbone than they do in the relaxed state, largely filling the space between myosin filaments (Figs. 1 *b* and 2 *b*). The loss of helical order in the H-zone on going from the relaxed to the rigor state is illustrated at high magnification in Fig. 2.

As discussed by Clarke et al. (1986), these structural changes in unattached cross-bridges are unlikely to be due to ATP-dependent changes in ionic strength, free $[Mg^{2+}]$ or myosin phosphorylation. ATP concentrations of only 0.1 mM or less are sufficient to produce ordered cross-bridge arrays (Clarke et al., 1986; Schlichting and Wray, 1986; Frado and Craig, 1989), and large changes in ionic strength and $[Mg^{2+}]$ do not affect the helical order (Schlichting and Wray, 1986). Phosphorylation of myosin is unlikely to occur in the relaxing solution (which contains 5 mM EGTA) because free Ca^{2+} is essentially absent and intrinsic light chain kinase would therefore be expected to be inactive; in addition, if phosphorylation were to occur in relaxing solution, it would be expected to produce the opposite effect (i.e., disorder) to that observed, by analogy with tarantula striated muscle filaments (Craig et al., 1987; Panté et al., 1988) and vertebrate smooth muscle thick filaments (Ikebe and Ogihara, 1982). The loss of helical order is also unlikely to be an effect of pH change, which may have been the case in x-ray studies of live muscles put into rigor (Haselgrove, 1975; cf., Wray and Holmes, 1981) because Schlichting and Wray (1986) found that the relaxed state was insensitive to large changes in pH, and the relaxing and rigor solutions used to bathe the filament lattice in our skinned specimens were each buffered at pH 7.0.

We conclude that the structural changes we observe (outward movement of the cross-bridges and loss of helical order in the H-zone and actin-based order in the

[1]PVP was included to prevent swelling of the muscle lattice after skinning (Padrón and Huxley, 1984; Magid and Reedy, 1980): at high concentrations PVP also has a significant cryoprotectant effect, but at the 2% level used, such effects are negligible (see Robards and Sleytr, 1985).

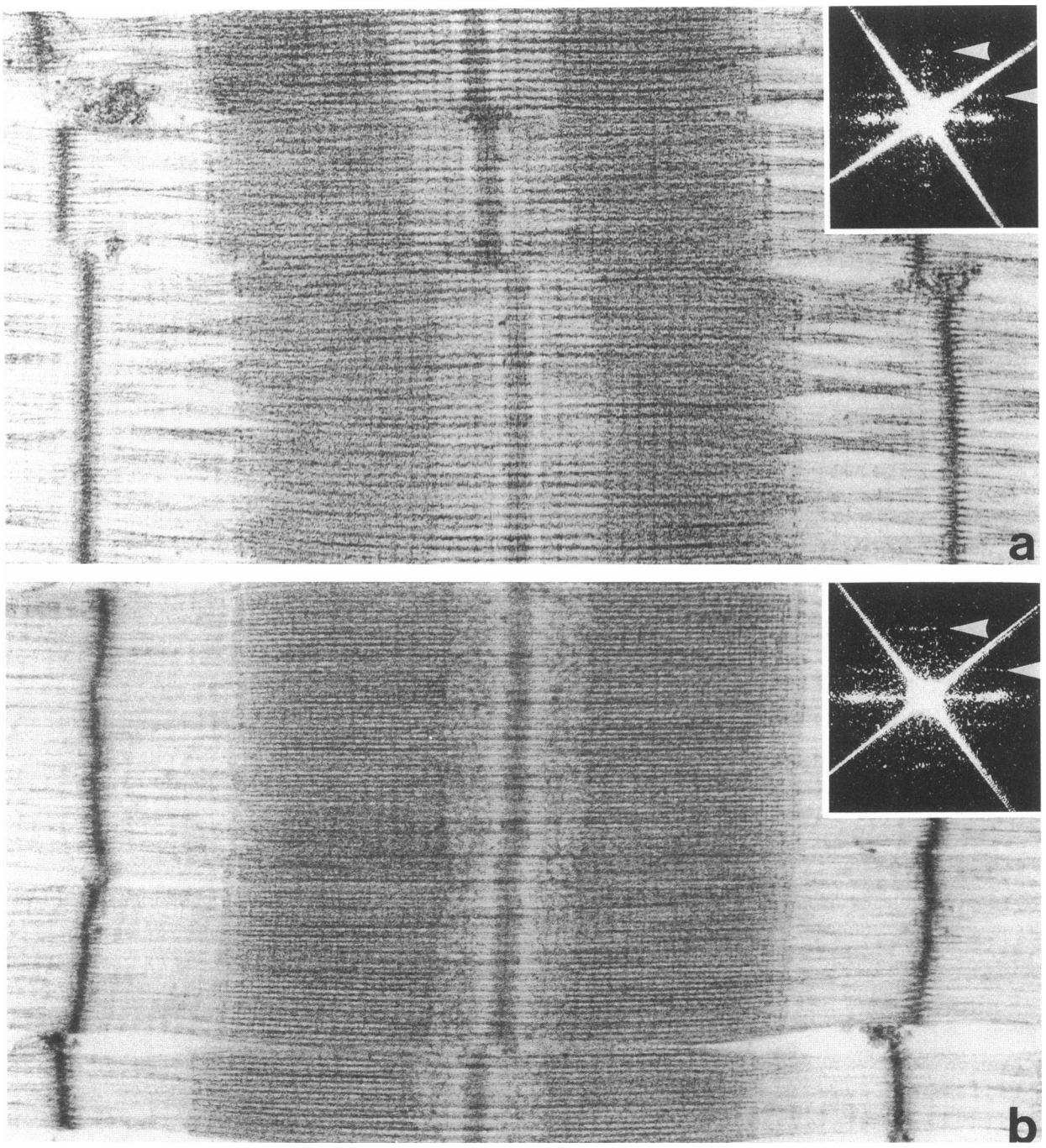

FIGURE 1 Longitudinal sections of rapidly frozen, freeze-substituted, demembranated frog sartorius muscles. (*a*) In relaxing solution; (*b*) in rigor solution. Note the striking difference between the uniform helical array of cross-bridges seen in (*a*) along the whole thick filament (best viewed by holding the micrograph at a glancing angle and viewing along the helical tracks; see also Fig. 2 *a*), compared with two distinct regions in *b*, one of local disorder (in the nonoverlap region of the A-band; see also Fig. 2 *b*), the other of local order (in the overlap region). Insets show optical diffraction patterns of A-bands of similar specimens (the muscle axis from which these diffraction patterns were obtained was vertical). Upper and lower arrows in *a* correspond respectively to the 14.3- and 43-nm repeats respectively in x-ray diffraction patterns of relaxed muscle. Upper and lower arrows in *b* correspond respectively to the 14.4- and 37-nm x-ray reflections from rigor muscle. Freeze substitution was with 5% osmium in acetone (*a*) and in methanol (*b*). Magnifications, 60,000×.

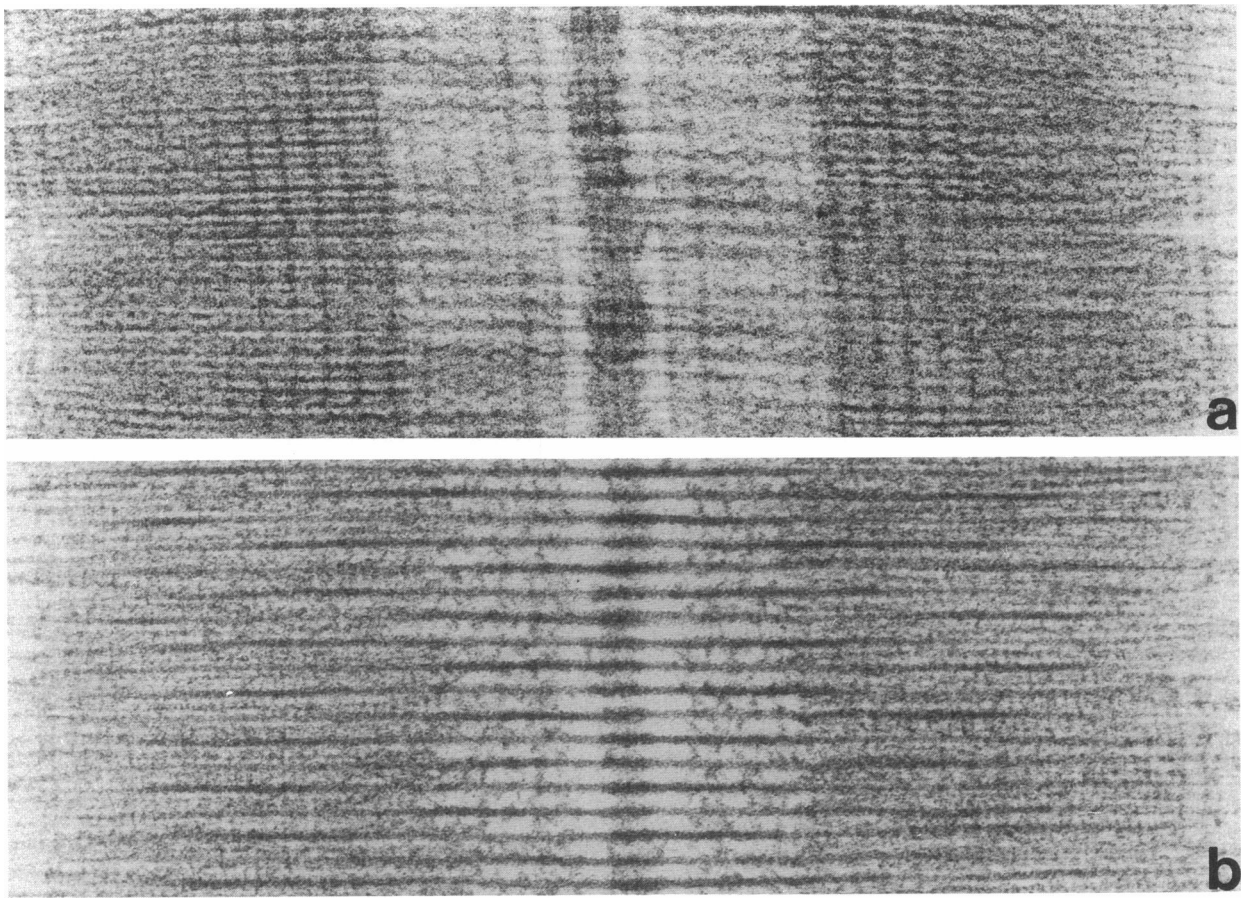

FIGURE 2 High magnification comparison of A-bands to show ATP-dependent change in cross-bridge order in H-zones. (*a*) Relaxed state showing helical cross-bridge order; and (*b*) rigor state, showing loss of helical order. Magnifications, 110,000×.

overlap zone) are due specifically to loss of ATP from the active site on the myosin heads.

DISCUSSION

Changes in the surface array of myosin heads on the thick filaments of striated muscle after removal of ATP have been reported by a variety of techniques. Several studies using negative staining of isolated native or synthetic thick filaments from different invertebrate muscles suggest that cross-bridges not attached to actin are disordered (or move away from the filament backbone) compared with those in relaxed thick filaments (Craig and Padrón, 1982; Vibert and Craig, 1985; Levine et al., 1985, 1986; Clarke et al., 1986; Frado and Craig, 1989), and several of these studies show that this effect is reversible. However, these experiments do not directly give information on changes occurring in the intact filament lattice and are subject to possible negative staining artifacts. Image analysis of frozen hydrated transverse thin sections of skeletal muscle shows that myosin filaments in the nonoverlap zone appear to broaden on entering rigor, suggesting that on deprivation of ATP cross-bridges in situ move outwards (Trus et al., 1989). This finding is consistent with the results shown in Fig. 1, but it does not directly reveal any change in the ordering of the cross-bridges. X-Ray studies of frog (Haselgrove, 1975; Poulsen and Lowy, 1983; Schlichting and Wray, 1986) and *Limulus* (Wray, 1984) thick filaments in the intact filament lattice show that ATP depletion causes loss of myosin layer lines (coming from the helical arrangement of myosin cross-bridges) in muscles that have been stretched so that there is little or no overlap between thick and thin filaments, and that there is an increase in diffuse scatter (Poulsen and Lowy, 1983), which is ascribed to an increase in the number of disordered heads. Although these x-ray experiments do not directly give information on unattached cross-bridges at physiological sarcomere lengths, they agree well with the results on loss of helical

order in the H-zone shown in Figs. 1 and 2, where the sarcomere length is close to that occurring in vivo.

Spin-label studies of glycerinated rabbit muscle in the absence of ATP at different sarcomere lengths indirectly suggest that in the overlap zone, myosin heads are oriented and immobile, whereas in the nonoverlap zone they are highly disordered (Thomas and Cooke, 1980; Barnett and Thomas, 1984), in good agreement with the findings presented here; the spin-label data further suggest that the disordered heads are mobile on the microsecond timescale. There is an apparent disagreement, however, in the case of relaxed muscle, where spin-label studies (Thomas et al., 1980; Thomas and Cooke, 1980; cf., Reedy and Cooke, 1989) do not detect the ordering of cross-bridges known to be present from x-ray diffraction and electron microscopic data. Other spectroscopic techniques (Nihei et al., 1974; Taylor, 1976; Burghardt et al., 1984) also show no difference between cross-bridges in the presence and absence of ATP in glycerinated rabbit psoas muscle stretched to nonoverlap. Possible reasons for some of the discrepancies between spectrocopic studies on the one hand and x-ray and negative staining studies on the other have been discussed by Barnett and Thomas (1984) and by Clarke et al. (1986).

The most direct visual way of observing structural changes in myosin cross-bridges in the intact filament lattice is by electron microscopy of sectioned or freeze-fractured specimens. However, preservation of the helical array of myosin heads on the thick filaments of intact relaxed muscles has not been achieved by standard electron microscopic sectioning methods. This is probably due to disruption of the in vivo structure by chemical fixation and embedding procedures (Reedy, 1976; Reedy et al., 1983), and to the fact that, in the case of rabbit psoas muscle, which has been the subject of many electron microscopic studies of vertebrate striated muscle, the helical array of myosin heads in relaxed muscle is disordered at low temperature (Wray, 1987; Wakabayashi et al., 1988). A meaningful comparison between myosin filament structures in relaxed and rigor muscles has therefore not been possible.

We have shown, on the other hand, that rapid cryofixation followed by freeze substitution (van Harreveld and Crowell, 1964; Heuser et al., 1979) preserves the helical order of the myosin heads in relaxed frog thick filaments in close to the in vivo state (Fig. 1 *a*; Padrón et al., 1988 *a* and *b*; cf., Tsukita and Yano, 1985). This is demonstrated by comparison of optical diffraction patterns of freeze-substituted frog muscle (Fig. 1) with x-ray diffraction data from relaxed, intact frog muscle (Huxley and Brown, 1967; Magid and Reedy, 1980; Padrón and Huxley, 1984). Preservation of the myosin helix has also recently been demonstrated in freeze fracture images of rapidly frozen relaxed muscle (Ip and Heuser, 1983; Cantino and Squire, 1986). The preservation of the ordered cross-bridge array of relaxed muscle in a state approximating that occurring in vivo by rapid freezing suggests that the structure observed in the absence of ATP should also be close to reality. In this case, nonoverlap cross-bridges project far from the filament backbone and lose their helical order, whereas cross-bridges overlapping with thin filaments show ordering on the actin helix as indicated by the ~35-nm repeat of the oblique stripes of rigor muscle (Fig. 1 *b*, *inset*; cf., Huxley, 1963; Wray et al., 1978). Electron microscopy of freeze-substituted, intact sarcomeres (Figs. 1 and 2) not only appears to provide reliable images, but also allows discrimination between the structure of overlap and nonoverlap cross-bridges in the same thick filaments, information that is not directly available by x-ray diffraction, by negative staining of isolated filaments, or by spectroscopic techniques. Our results thus provide a direct, visual demonstration of the changes in cross-bridge order in whole muscle previously inferred from x-ray studies of muscle stretched to nonoverlap. They are also consistent with the conclusion from spectroscopic measurements that unattached cross-bridges are disordered in the absence of ATP (Thomas and Cooke, 1980; Barnett and Thomas, 1984), and suggest that the ATP-dependent changes observed in isolated filaments by negative staining are not an artifact of staining and also occur in the intact filament lattice.

The effects of ATP on the myosin cross-bridge array that we have observed probably reflect a general property of myosin filaments and may be of relevance to the cross-bridge cycle, as discussed by Clarke et al. (1986). Our results show that the helical cross-bridge order in relaxed muscles is dependent on the presence of ATP (cf., Poulsen and Lowy, 1983; Schlichting and Wray, 1986; Frado and Craig, 1989); it has further been suggested that ordering may specifically require that the ATP be present in the hydrolyzed state (ADP.Pi) on the myosin heads (Wray, 1987; cf., Wakabayashi et al., 1988; Frado and Craig, 1989). The basis of this order may be specific interactions between the thick filament backbone and the myosin head or S2 (Poulsen and Lowy, 1983; Crowther et al., 1985), or head–head interaction between adjacent 14-nm levels of cross-bridges (Crowther et al., 1985). The close association of the myosin heads with the thick filament suggested by their ordering on the myosin repeat may contribute to the low level of ATP consumption in relaxed muscle by restricting interaction of myosin heads with actin in the filament lattice, as suggested by Crowther et al. (1985).

Loss of helical order in the H-zone of rigor muscle is most simply interpreted as a weakening of the binding of the myosin heads to the thick filament backbone in the absence of ATP. The loosened heads are apparently

highly mobile as indicated by spin-label data (Barnett and Thomas, 1984), but they can, after their release from the thick filament backbone, become immobilized and ordered again if actin filaments are available for binding (Haselgrove, 1975). The heads of regulated myosin filaments also lose their helical order under activating conditions, suggesting a weakening of their interaction with the filament backbone, which may facilitate their interaction with actin during contraction (Vibert and Craig, 1985; Craig et al., 1987; Panté et al., 1988; Frado and Craig, 1989). Whether such loss of helical order and outward cross-bridge movement occurs in the H-zone during contraction of muscles which are not myosin-regulated is uncertain (Huxley, 1972; Huxley et al., 1980; Yagi and Matsubara, 1980; Wray and Holmes, 1981) but rapid freezing of contracting muscle offers promise of answering this question directly.

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