Structural Changes that Occur in Scallop Myosin Filaments upon Activation

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ABSTRACT   Myosin filaments isolated from scallop striated muscle have been activated by calcium-containing solutions, and their structure has been examined by electron microscopy after negative staining. The orderly helical arrangement of myosin projections characteristic of the relaxed state is largely lost upon activation. The oblique striping that arises from alignment of elongated projections along the long-pitched helical tracks is greatly weakened, although a 145 Å axial periodicity is sometimes partially retained. The edges of the filaments become rough, and the myosin heads move outwards as their helical arrangement becomes disordered. Crossbridges at various angles appear to link thick and thin filaments after activation. The transition from order to disorder is reversible and occurs over a narrow range of free calcium concentration near pCa 5.7. Removal of nucleotide, as well as dissociation of regulatory light chains, also disrupts the ordered helical arrangement of projections.

We suggest that the relaxed arrangement of the projections is probably maintained by intermolecular interactions between myosin molecules, which depend on the regulatory light chains. Calcium binding changes the interactions between light chains and the rest of the head, activating the myosin molecule. Intermolecular contacts between molecules may thus be altered and may propagate activation cooperatively throughout the thick filament.

Knowledge of the conformations adopted by the head region of the myosin molecule is essential for understanding how myosin–actin interactions produce force (1). Studies of myosin filament structure in relaxed striated muscles by x-ray diffraction (2–5), and of relaxed isolated filaments by electron microscopy and image analysis (6–14), have defined the helical symmetry of the surface array of myosin projections in a number of muscle types. In addition, these investigations reveal the structure of the projections at low resolution: they usually appear as elongated units lying close to the surface of the filament backbone, are oriented more nearly parallel than perpendicular to the filament axis, and are skewed so that they run approximately along the principal right-handed helical tracks of pitch 430–485 Å. The number and size of the projections indicate that each contains two myosin heads, but only in tarantula muscle have the heads been resolved, apparently splayed apart from each other (11, 14).

In contracting vertebrate striated muscles, the helical arrangement of myosin heads becomes substantially disordered

as the heads interact with actin (2, 15, 16). This disordering apparently does not occur when there is no overlap with actin (17), presumably because vertebrate skeletal myosin does not itself sense activation by calcium. Myosins from many muscle and nonmuscle cells are, however, activated by increased sarcoplasmic calcium levels (18–21) and may be expected to change structure upon activation independently of their interaction with actin. In regulated muscle myosins, two classes of activation mechanism have been identified. One involves Ca^{2+}-dependent phosphorylation of myosin light chains (21–23), and the other depends on direct Ca^{2+} binding to a site on the myosin head and also involves light chains (18, 24–27). Both forms of myosin regulation show cooperativity between the two heads of each myosin molecule (27–30); in addition, when molluscan myosin is assembled into filaments, intermolecular interactions give rise to a highly cooperative response to Ca^{2+} activation (28). The isolation of well-preserved myosin filaments from relaxed muscle (7, 9, 11, 12) opens the possibility of studying these mechanisms of activation by electron microscopy; myosin and actin filaments can be sep-
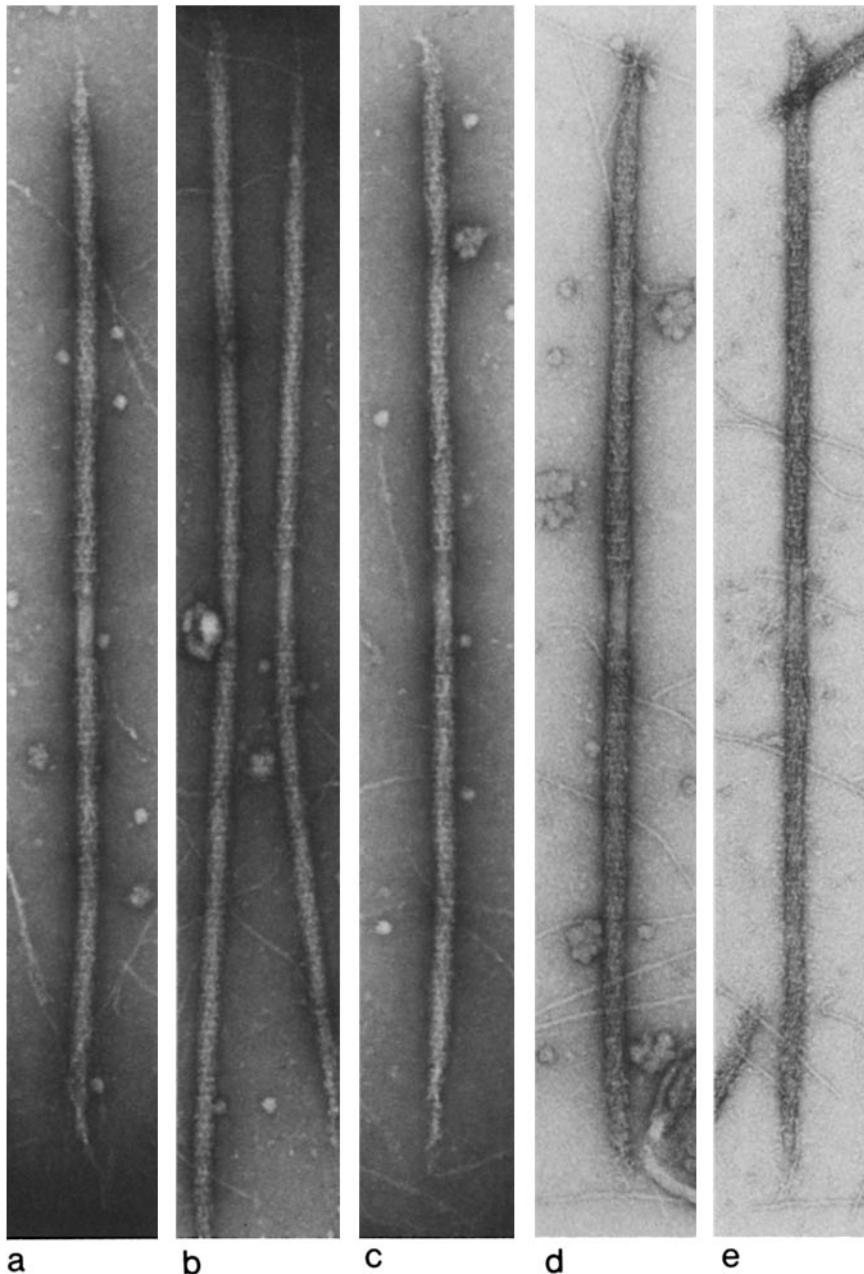
FIGURE 1 Myosin filaments isolated from scallop striated muscle and maintained in relaxing solution until negatively stained. (a–c) Filaments suspended over a hole in carbon film, stained with 4% UrAc. (d and e) Filaments supported on thin carbon film and stained with (d) 2% UrAc or (e) 2% UrAc + 5 mM sucrose. × 73,000.

a   b   c   d   e

arated from each other, and the Ca^{2+} concentration or degree of light chain phosphorylation can be controlled.

We have examined how filaments isolated from scallop striated muscle respond to activation by Ca^{2+} ions, as well as to nonphysiological means of inducing activation or myosin–actin binding (removal of nucleotide or of regulatory light chains [18, 24, 25, 27]). We have found that negative staining with uranyl acetate produces consistent electron microscope images of two distinct structural states of the myosin projections: a relaxed state in which the projections are ordered, and an activated state in which they are disordered. A preliminary report of this work has been given elsewhere (31).

MATERIALS AND METHODS

We prepared detergent-skinned fiber bundles from the striated adductor muscle of the sea scallop *Placopecten magellanicus* as described previously (12). Filament suspensions were obtained by brief and gentle homogenization of the skinned bundles in either relaxing or rigor solutions (see Results). To maintain the filaments under defined ionic conditions until negative staining, the normal

rinse with ammonium acetate solution was omitted; instead, all solutions had acetate (Ac)[1] as the major anion (11, 14). Relaxing solution contained 0.1 M NaAc, 3 mM $MgAc_2$, 2 mM Na_2ATP, 1 mM EGTA, 2 mM imidazole, pH 7.0, at 4°C. For rigor solution, Na_2ATP was omitted. We made activating solutions with various free Ca^{2+} concentrations by adding 0.15–1.0 mM $CaAc_2$ to relaxing solution and readjusting to pH 7.0. Regulatory light chains were removed by a desensitizing solution containing 0.1 M NaAc, 10 mM EDTA, 2 mM imidazole, pH 7.0, at 4°C. (Light chain removal was not measured but is expected, on the average, to amount to one light chain per myosin at this temperature [27].) We calculated the concentrations of free Ca^{2+} and of other ions and complexes by the method of Perrin and Sayce (32), using a program for a VAX 11/780 (Digital Equipment Corp., Maynard, MA) kindly donated by R. Padrón (MRC Laboratory of Molecular Biology, Cambridge, U.K.) (see reference 33). Association constants (at 4°C) for the various ionic species were taken from references 28 and 34. In the various activating solutions, the pCa values ranged from 5.0 to 7.1, with ~0.9 mM free Mg^{2+}.

Negative staining of filament suspensions was performed normally (12) using ice-cold solutions. A drop of filament suspension was placed on either a holey carbon or thin intact carbon film, supported by a 400-mesh copper grid, for ~15 s (thin carbon films used within 2–3 d of being made were suitably hydrophilic). The grid was then rinsed with eight drops of the required solution

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[1] *Abbreviations used in this paper.* Ac, acetate; UrAc, uranyl acetate.

(relaxing, activating, rigor, or desensitizing), and the last drop was allowed to remain on the grid for as long as had been found necessary in repeated trials for most filaments to have a similar appearance (see Results). This period ranged from a few seconds (activation) to 30 min (rigor induction); we kept samples cool and minimized evaporation by placing the steel forceps in which the grid was held on ice, and also on some occasions by working in a room with controlled high relative humidity. The filaments were then stained with six drops of 2–4% aqueous uranyl acetate, sometimes with added 5 mM sucrose; excess stain was removed with filter paper, and the grids were allowed to dry at room temperature. Grids were examined at 80 kV with a 30- or 60-μm objective aperture and anticontamination cold finger in either a Philips EM 301 or a JEOL 100-CX electron microscope. Micrographs were recorded at a nominal magnification of 25,000 under standard high-dose conditions.

RESULTS

Relaxed Filaments

Scallop thick filaments, isolated and maintained in relaxing solution until negative staining, usually show an ordered helical surface array of projections (Fig. 1; compare reference 12). Their appearance is similar whether they are suspended in stain over holes (Fig. 1, a–c) or supported by a thin carbon film (Fig. 1, d and e). A "spread" configuration (in which myosin heads are splayed out from the filament backbone and bind to the carbon support film) has been described for vertebrate skeletal filaments (e.g., reference 35) and is occasionally seen also for relaxed scallop filaments on carbon. Long-range periodicities, as judged by optical or computed transforms, are better preserved when the filaments are suspended over holes in a thick sheet of uranyl acetate (compare reference 12); fine detail, however, seems to be revealed more readily (usually at the expense of long-range order) when the filaments are embedded in thinner stain on carbon films, especially when sugars (e.g., 5 mM sucrose) are added to the
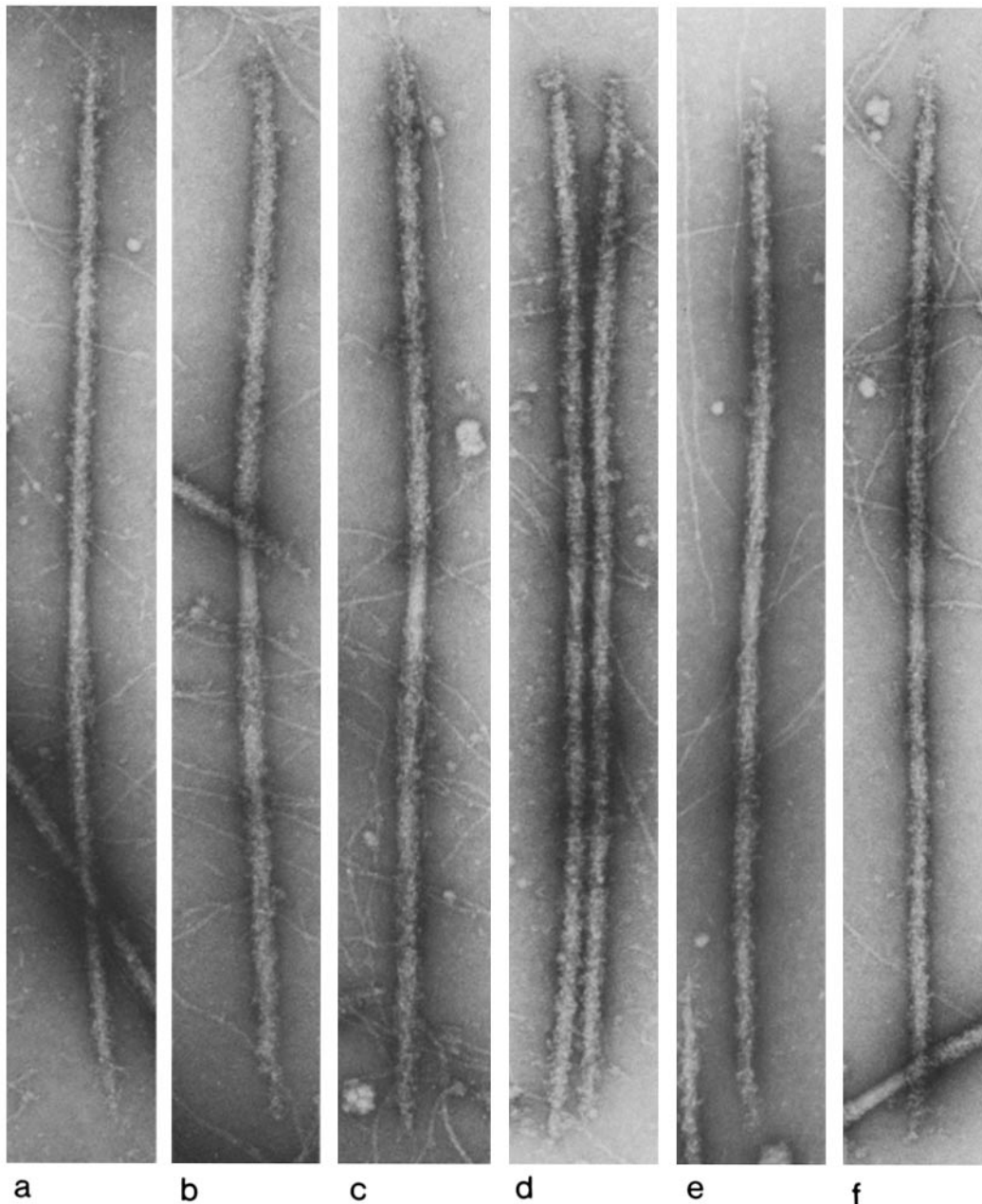

a b c d e f

FIGURE 2 Scallop myosin filaments washed with activating solution (pCa 5.0) for ~10 s before staining were suspended over holes and stained with 2% UrAc. (a–c) Projections are largely disordered. (d–f) Filaments retain some 145 Å periodicity. × 73,000.

stain (compare Fig. 1, a–c with d and e).

The characteristic axial and oblique periodicities of the surface array arise from elongated projections, spaced at 145-Å intervals, that are aligned along the helical tracks of pitch ~485 Å (12). These projections probably correspond to unresolved pairs of myosin heads, but at this resolution it is not clear whether two heads lie approximately parallel and in register within each projection (12), or whether the heads from each myosin molecule splay apart axially as reported for tarantula thick filaments (11, 14).

Activated Filaments

When relaxed filaments on an electron microscope grid are washed briefly with calcium-containing activating solutions (pCa 5.0) before negative staining, the appearance of the surface array of projections is altered. In a typical case, the oblique striping is greatly weakened, but some 145 Å axial periodicity is often retained (Fig. 2, a–f). The edges of the

filaments become rough, the overall diameter of the filament increasing by ~100 Å. Thus the myosin heads appear to move outward, and their helical arrangement becomes disordered upon activation. Activated filaments supported on carbon often show myosin heads splayed further out onto the carbon

TABLE I.   Response of Scallop Filaments to Calcium Activation

[Ca]/[EGTA]	Approximate pCa	Filament appearance
0.15	7.1	R
0.35	6.6	R
0.5	6.4	R
0.6	6.2	R
0.8	5.8	R/A
0.9	5.5	R/A
1.0	5.0	A

R, filaments appear ordered (similar to those in relaxing solution). A, filaments appear disordered (similar to those in pCa 5 activating solution).

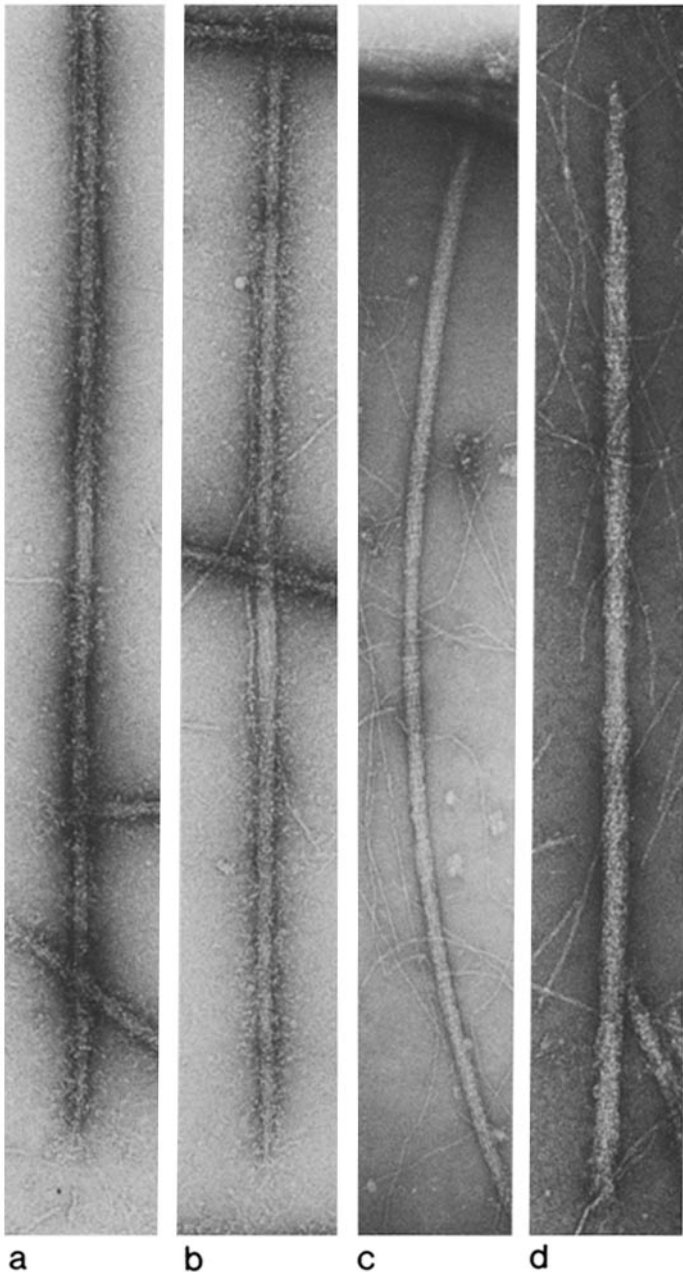a   b   c   d

FIGURE 3   (a and b) Activated filaments (pCa 5.0) supported on carbon, stained with 2% UrAc + 5 mM sucrose. (c) The filament was activated (pCa 5.0) for 30 s, then relaxed for 30 s more before staining. It was suspended over a hole and stained with 4% UrAc (the filament was distorted by shrinkage of broken stain film). (d) 10-s activation followed by 10-s relaxation before staining. The filament was supported on carbon and stained with 2% UrAc + 5 mM sucrose. × 73,000.

surface (Fig. 3, *a* and *b*), but the filament backbone thus revealed shows little periodicity.

The degree of disorder of the projections in activated filaments is somewhat variable (Fig. 2, *a–f*), but fields of filaments could be easily recognized in "blind" experiments as relaxed or activated. It seems unlikely that these different appearances represent successive stages of activation (which probably occur on a millisecond time scale), but rather they reflect the fact that our comparatively slow method of staining traps varied dispositions of heads that have been mobilized.

Activation at different levels of free Ca^{2+} ions shows that the transition from the ordered relaxed structure to the disordered activated structure occurs over a narrow range of concentrations near pCa 5.7 (Table I). This concentration is close to that at which scallop myofibrillar ATPase and fiber tension are sharply activated (28), suggesting that the disorder of the filament surface array is a specific response to calcium binding. SDS PAGE (not shown) of filament homogenates incubated in relaxing or activating solutions for 12 min at 4°C showed indistinguishable band patterns, indicating that nonspecific Ca^{2+}-induced proteolysis is not responsible for disorder on activation.

When filaments are activated at pCa 5 on the grid and then rinsed with relaxing solution before staining, the surface array sometimes regains a well-ordered appearance, although this is not consistently seen (Fig. 3, *c* and *d*). Whereas activation is rapid (filaments stained a few seconds after activation show disordered surface arrays), relaxation is significantly slower under our conditions: even when the last drop of relaxing solution is left on the grid for 5–10 min, some filaments remain disordered.

The filament homogenates contain many thin filaments as well as thick filaments, and after activation some myosin-actin binding is observed (Fig. 4). (Unlike those of arthropod muscles [7], scallop muscle homogenates show little attachment or alignment of thick and thin filaments under relaxing conditions.) Crossbridges at various angles appear to link thick and thin filaments across a surface-to-surface gap of 100–200 Å (Fig. 4), an arrangement that may represent interactions between actively sliding filaments.

Effects of Nucleotide

When relaxed filaments are washed repeatedly on the grid with rigor solution before staining, the projections also become disordered (Fig. 5, *a* and *b*). The time scale of the transition is, however, ~1,000 times slower than in the case of Ca^{2+} activation: some filaments appear ordered even when the last drop of rigor solution remains on the grid for 30 min before staining. (Control experiments show that filaments kept in a drop of relaxing solution for 30 min retain their ordered appearance.) A more complete "rigor state"was achieved when filament homogenates were obtained from skinned fiber bundles that had been kept in rigor solutions for 12 h or more (Fig. 5, *c* and *d*). In these preparations, bundles of closely associated thick and thin filaments are found. The thick filaments appear to have a disordered surface array, and uniformly and acutely angled crossbridges between thick and thin filaments can be seen.

In contrast to the slow disordering associated with removal of ATP, relaxation from rigor is rapid. After as little as 10 s washing with relaxing solution, the bundles of thick and thin filaments from rigor fibers are substantially dissociated, and
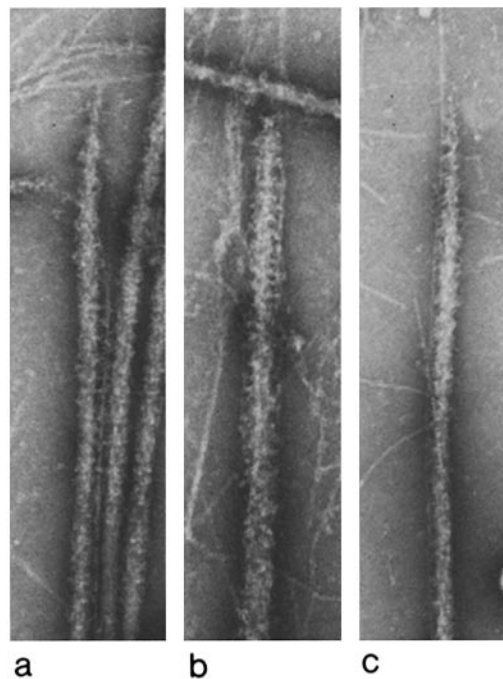

a   b   c

FIGURE 4 (a–c) Crossbridges connecting thick and thin filaments after activation (pCa 5.0). The filaments were suspended over a hole and stained with 4% UrAc. × 73,000.

the surface array on the thick filaments appears ordered. After ~30 s the homogenates are indistinguishable from those prepared under relaxing conditions (Fig. 5, *e* and *f*).

Removal of Regulatory Light Chains

The rapid and consistent relaxation induced by adding ATP to rigor filaments (see above) provides a reliable control for studying the effects of light chain removal. When filament homogenates from rigor fibers are washed with EDTA-containing solutions for 10 min at 4°C (a treatment that is expected to remove, on the average, one regulatory light chain per myosin molecule [27]), and the grids are then washed with relaxing solution before staining, the filaments do not relax but retain the disordered rigor appearance (Fig. 5g).

DISCUSSION

Electron microscopy of isolated scallop myosin filaments that have been treated with Ca^{2+}-containing solutions offers one of the most direct approaches to visualizing the mechanism of activation of a regulated myosin in the absence of interaction with actin. The studies described here represent a preliminary investigation of this process but are limited in both temporal and spatial resolution. Negative staining with unbuffered aqueous uranyl acetate (UrAc) preserves two structural states of the myosin projections (ordered and disordered) with a consistency that suggests that defined ionic conditions (for example, high or low Ca^{2+} concentrations) are maintained until the stain effectively "fixes" the protein structure. Although the nonphysiological transitions induced by removal of ATP or regulatory light chains are probably slow enough for distinct structural states to be trapped in this manner (36, 37), more rapid fixation would be required to trap the steps of activation (which in intact scallop muscle occur almost as rapidly as in frog muscle, i.e., on a millisecond time scale
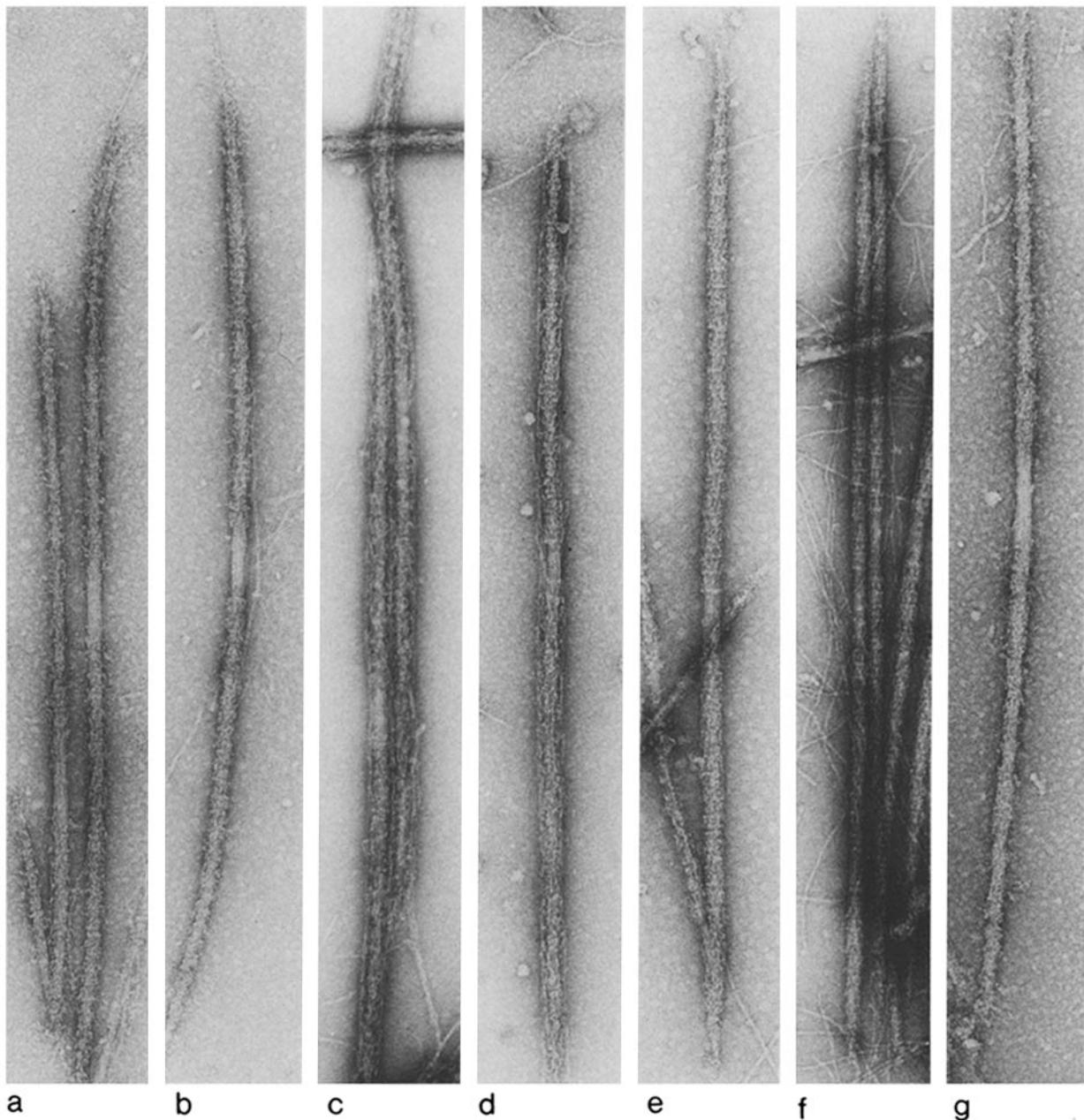
FIGURE 5   (a and b) Filaments washed with rigor solution for 30 min before staining were supported on carbon and stained with 2% UrAc. (c and d) Crossbridged thick and thin filaments from a rigor fiber bundle were supported on carbon and stained with 2% UrAc. (e and f) Rigor homogenates (as in c and d) washed with relaxing solution for 1 min before staining. Filaments were supported on carbon and stained with 3% UrAc. (g) Rigor homogenate (as in c and d) washed with desensitizing solution (see text) for 5 min and then with relaxing solution for 1 min before staining. Filaments were supported on carbon and stained with 3% UrAc. × 73,000.

[38]). Thus, our methods provide a time-averaged picture of fully activated filaments.

Qualitatively similar order–disorder transitions are observed in scallop myosin filaments in response to Ca^{2+} binding (Figs. 1–4) and removal of regulatory light chains (Fig. 5g). The effects of Ca^{2+} appear to be specific, since the transition is reversible and occurs over a narrow range of free Ca^{2+} concentrations (in the presence of millimolar free Mg^{2+}) close to that at which scallop myofibrillar ATPase and fiber tension are sharply activated (28). The physiological form of regulation requires the presence of regulatory light chains that maintain molluscan myosin in an "off" state in the absence

of Ca^{2+}. When these small subunits are removed, ATPase and tension are activated (24, 25, 27). The similar structural and enzymatic transitions that occur in myosin filaments upon Ca^{2+} binding and light chain removal may reflect the fact that in both cases interactions between the light chain and head that maintain the off state are disrupted. The importance of the regulatory light chains in stabilizing the structure of the "neck" region of scallop myosin has already been inferred from studies of individual molecules (39) and of decorated actin (40). (The homologous light chains probably also contribute to the structural integrity of the neck region in vertebrate striated [40–42] and smooth muscle myosins [43].) In

the case of scallop myosin, our observations indicate that the regulatory light chains also play a structural role in maintaining the order of the helically arranged projections on relaxed myosin filaments.

The interactions that maintain the ordered helical arrangement are only now beginning to be understood. The two heads of myosin appear to have no preferred spatial relationship to one another or to the myosin rod in isolated molecules (39, 44, 45). The orderly alignment of heads on relaxed filaments therefore presumably depends on specific intermolecular interactions, for example between myosin heads from molecules 145 Å apart axially, or between heads and adjacent rods in the filament backbone. It is not yet clear whether there are intermolecular interactions that are a specific feature of regulated myosin filaments. It is notable, however, that the ordered arrays of projections appear to be most stable on filaments from myosin-regulated muscles (6–12, 14). Although ordered projections on native relaxed filaments were first demonstrated for unregulated vertebrate striated muscle myosin (2), in these filaments some or all relaxed heads may also be highly mobile (46, 47), which may account for the difficulty in preserving the order of projections for microscopy (13). Measurements of head motion have not yet been made on regulated myosins in the off state. When activated by Ca^{2+}, scallop filaments lose their oblique striping but often retain a clear 145-Å periodicity (Fig. 2, d–f), which suggests that different classes of contacts may stabilize the helical and transverse alignment of relaxed projections in these regulated filaments. Release of the helical contacts alone might allow the heads of regulated filaments to interact more readily with actin.

In contrast to the effects of Ca^{2+}, the disordering of the projections on removal of nucleotide does not appear to be a specific property of calcium-sensitive myosin filaments. Similar disorder apparently also occurs in filaments from vertebrate striated muscle (48, 49), where myosin does not adopt an off conformation (20, 24), as well as in *Limulus* muscle (50), whose myosin is regulated by light chain phosphorylation (23). All myosins probably undergo similar changes in head conformation upon binding or releasing nucleotide; light chains may, however, be important in stabilizing these conformations.

Activation of scallop myosin involves some rearrangement of the light chains, which includes movement of the SH ("essential") light chain in a region near the head–rod junction (51–54). These movements have so far been detected by their effects on cross-linking between light chains and appear to be beyond the level of resolution of electron microscopy of myosin molecules contrasted by shadowing (54). Higher resolution studies of crystalline head aggregates (41, 55) may be needed to visualize these light chain motions. The results of these small conformational changes are, however, dramatically expressed in the structural transitions of the intact myosin filaments described here. Similarly, phosphorylation of a single site on the light chains of tarantula muscle myosin is associated with disorder of the helical arrangement of heads in myosin filaments (56). Rapid methods of fixing filament structure for electron microscopy, including freezing (57–59), may allow the activation mechanisms of these native myosin assemblies to be examined in detail.

Activation is known to be a highly cooperative process in scallop myosin filaments (28), reflecting a general property of interacting protein systems, which is shown also by the tro-

pomyosin/troponin assembly that regulates thin filaments in other muscles (e.g., references 60 and 61). The mechanics of myosin activation are not yet understood but may be pictured as a cascade of protein–protein interactions where cooperativity increases at higher levels of organization. Thus localized light chain movements alter the structure of the "regulatory domain" (36, 62, 63) of the myosin head; interdomain motions transmit signals to the actin- and nucleotide-binding sites within each head and communicate between sites in both heads (53, 54); and changes in intermolecular contacts may propagate activation throughout the thick filament (28).

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