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Millisecond Time-Resolved Changes Occurring in Ca^{2+}-Regulated Myosin Filaments upon Relaxation

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Contraction of many muscles is activated in part by the binding of Ca^{2+} to, or phosphorylation of, the myosin heads on the surface of the thick filaments. In relaxed muscle, the myosin heads are helically ordered and undergo minimal interaction with actin. On Ca^{2+} binding or phosphorylation, the head array becomes disordered, reflecting breakage of the head–head and other interactions that underlie the ordered structure. Loosening of the heads from the filament surface enables them to interact with actin filaments, bringing about contraction. On relaxation, the heads return to their ordered positions on the filament backbone. In scallop striated adductor muscle, the disordering that takes place on Ca^{2+} binding occurs on the millisecond time scale, suggesting that it is a key element of muscle activation. Here we have studied the reverse process. Using time-resolved negative staining electron microscopy, we show that the rate of reordering on removal of Ca^{2+} also occurs on the same physiological time scale. Direct observation of images together with analysis of their Fourier transforms shows that activated heads regain their axial ordering within 20 ms and become ordered in their final helical positions within 50 ms. This rapid reordering suggests that reformation of the ordered structure, and the head–head and other interactions that underlie it, is a critical element of the relaxation process.

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Muscle contraction is generated by cyclic interaction of myosin heads on the thick filaments with actin subunits in the thin filaments. This interaction, and hence contraction, is regulated by Ca^{2+} via the troponin–tropomyosin complex on the thin filaments (actin-linked regulation) or the myosin light chains on the thick filaments (myosin-linked regulation).[1–6] The regulatory movements of tropomyosin in actin-linked regulation are well understood. Electron microscopy (EM) reveals that tropomyosin blocks myosin-binding sites on actin in relaxed muscle, and moves so that these sites become exposed upon activation.[7,8] Time-resolved X-ray diffraction studies of contracting muscle demonstrate that movement of tropomyosin from its blocking position on activation is rapid (on the millisecond time scale) and occurs before myosin heads bind to actin.[9] This supports the view that tropomyosin movement in response to calcium binding to troponin is physiologically relevant and a prerequisite for head attachment.

The structural basis of myosin-linked regulation, which in many muscles occurs together with the troponin–tropomyosin system, is less well understood. Two types of myosin-linked regulation have been identified. In one, initiation of contraction requires binding of Ca^{2+} to the essential light chains, whereas the other requires phosphorylation of the regulatory light chains by Ca^{2+}-calmodulin activation of myosin light-chain kinase.[2,4,10,11] In relaxed muscles, the heads of regulated myosin filaments are helically ordered on the filament surface.[12–17] This ordered structure involves intramolecular head–head and head–tail interactions together with

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Abbreviations used: EM, electron microscopy; EGTA, 0.2 mM ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid.

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intermolecular interactions between myosin heads of axially neighboring molecules along the helical tracks.[18] These multiple weak interactions are thought to switch myosin off by inhibiting ATP product release and actin binding.[18,19]

Electron microscopy of both negatively stained and frozen-hydrated specimens shows that activation of Ca^{2+}- and phosphorylation-regulated thick filaments causes the helically ordered array of myosin heads to become disordered, reflecting a loosening of myosin head interactions.[16,20–23] However, the slow time scale of these experiments (seconds to minutes) does not reveal whether these changes are rapid enough to be physiologically relevant. Time-resolved X-ray studies of myosin activation (comparable to those on tropomyosin[9]), which would test the time scale and therefore physiological relevance of disordering, have not been reported. We developed an alternative, electron microscopic approach—millisecond time-resolved negative staining[24]—to study the time course of disordering in Ca^{2+}-regulated thick filaments (from the scallop[16]). We showed that uranyl acetate, routinely used as a negative stain, fixes macromolecular structure in less than 10 ms and can therefore be used to capture structural transients on the millisecond time scale. By placing stain and activating solution in series in a pipette (with an air gap separating the two), relaxed filaments on an EM grid can be briefly activated (10–70 ms) and then immediately stained (by rapidly flushing the grid with the contents of the pipette), thus arresting the activated structure at known millisecond time points for EM observation.[16,24] Using this procedure, we showed that Ca^{2+}-induced disordering of scallop filaments is fully developed within 30 ms,[16]

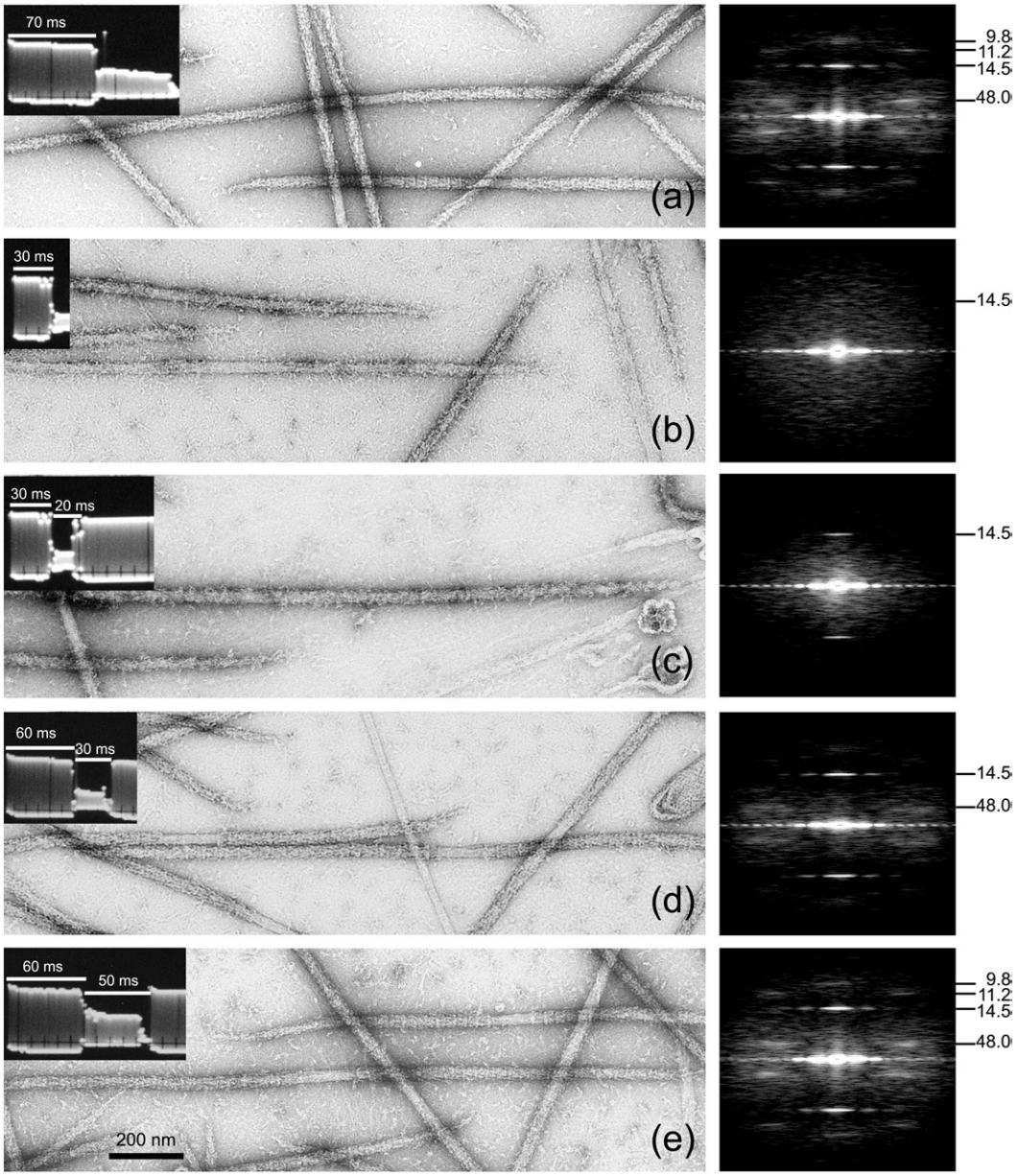


Fig. 1 (*legend on next page*)

well within the 90-ms time for development of peak twitch tension.[25] As with tropomyosin movement,[9] this time scale suggests that disordering is a key requirement of the physiological activation process. Disordering appears to result from breaking of the head–head and head–tail interactions that underlie the relaxed state.[23,26] The speed of disordering suggests that breaking of these interactions is also rapid, limited mainly by the kinetics of binding of Ca^{2+} to the heads.[16,27]

While these experiments provide insight into the time course of structural changes underlying myosin activation, we know little about the kinetics of the reverse process, when muscle relaxes. Our negative staining and cryo-EM studies have shown that removal of Ca^{2+} leads to the reappearance of helical order,[16,20] but as with activation, the slow time scale of these experiments leaves in doubt the physiological significance of this reversal. It is possible, for example, that reordering might occur by a slow 'annealing' process following dissociation of Ca^{2+}, and not be a requirement for switching off activity.

Here, we have tested the hypothesis that reordering occurs on the physiological time scale. We have done this using a variation on the time-resolved negative staining technique. By including a relaxing solution in the pipette, between the activating solution and the stain, filaments were first activated, then relaxed again for predetermined millisecond periods prior to rapid fixing with stain.

Figure 1a and b show relaxed and activated control filaments, respectively. Filaments flushed with relaxing rinse for a range of times (30–70 ms) were helically ordered, as judged both by eye and by the presence of layer lines in their Fourier transforms (Fig. 1a). This demonstrates that the rapid flushing procedure used in the time-resolved negative staining technique does not cause disorder, for example, by mechanical disruption.[16] The layer lines reveal the helical parameters of the scallop filament: the repeat of the main (7,3)[12,14] helical tracks is ~48 nm, while the axial spacing between neighboring 'crowns' of heads is ~14.5 nm (Fig. 1a).[12,14,15] The layer line at 11.2 nm indicates the presence of mass oriented along the (7,13) helical tracks,[12,14] while the meridional reflection at 9.8 nm reflects a small perturbation at alternating 14.5-nm levels of heads.[12,14]

When filaments were flushed with activating rinse, they rapidly became disordered, with their heads spread away from the filament backbone, reaching maximal disorder within 30 ms (Fig. 1b).[16] A weak meridional reflection at 14.5 nm remaining in the Fourier transform may represent vestiges of axial head ordering, or possibly visualization of the myosin axial periodicity in the filament backbone; similar residual reflections at 14.5 nm are seen in other species of filament with disordered heads (Ref. 17 and unpublished data). Longer activation times (50–70 ms) showed similar disorder, confirming our earlier findings.[16] We therefore used activation times from 30 to 70 ms for the reversal experiments.

To determine the rate of structural change following removal of Ca^{2+}, filaments were first activated (30–70 ms), then flushed with relaxing rinse (20–80 ms), and finally rapidly fixed with uranyl acetate. Within 20 ms, the heads had returned close to the

Fig. 1. Time course of head reordering on thick filaments following Ca^{2+} removal. Each panel shows a field of negatively stained scallop thick filaments, an oscilloscope trace (left inset) showing time in activating and/or relaxing rinse, and an averaged Fourier transform of 20 representative filaments at the right (numbers indicate spacing of main ~48-nm helical repeat (7,3) helices, axial spacing of head crowns (~14.5 nm), 11.2-nm spacing of the (7,13) helices, and 9.8-nm reflection coming from head perturbation (cf. Refs. 12 and 14). (a) Relaxed control: grid rinsed for 70 ms with relaxing rinse, then stained; (b) activated control: 30 ms activating rinse then stained; (c) 20 ms reversal: 30 ms activating rinse, 20 ms reversing rinse, then stained; (d) 30 ms reversal: 60 ms activating rinse, 30 ms reversing rinse, then stained; (e) 50 ms reversal: 60 ms activating rinse, 50 ms reversing rinse, then stained. The scale bar represents 200 nm. Methods: Scallop (*Placopecten magellanicus*) thick filaments were prepared from skinned striated adductor muscle and observed by time-resolved negative staining using solutions and techniques described previously.[16,24] Relaxing rinse was 100 mM NaAc, 3 mM $MgCl_2$, 0.2 mM ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM MgATP, 2 mM imidazole, 1 mM NaN_3 (pH 7.0). Activating rinse was the same, with $CaCl_2$ added to 0.4 mM total concentration (pCa ~4.0).[35] Reversing rinse was relaxing rinse containing total 4 mM EGTA. Experiments were carried out at ~21 °C. Time-resolved relaxation following activation was achieved by a variation of the technique previously described.[16,24] Three solutions (instead of two) were placed in the pipette tip, separated by air gaps. The first activated the filaments for 30–70 ms, producing similar disordering in all cases; the second was reversing rinse, which was applied for the next 20, 30, 50, 70 or 80 ms following activation to obtain 'snapshots' of the relaxation process. Finally, the structure present following these different relaxation periods was 'instantaneously' arrested by the third solution, 1% (w/v) uranyl acetate, which also served as a negative stain.[24] Most experiments were carried out by first making three to five grids each of the relaxed and activated controls and then three to five grids for the reversal. To minimize possible variation between grids, we also used the following sequence: one grid relaxed control, one grid activated control, then one grid reversal, the whole sequence being repeated three times. The results were the same with both approaches. Control experiments showed that the changes in order were not due to the minor changes in pH (due to proton liberation on binding of Ca^{2+} to EGTA) or ionic strength occurring as the filaments were flushed with the different solutions. Grids were examined at 80 kV in a Philips CM 10 electron microscope (FEI, Hillsboro, OR). Conventional dose micrographs were recorded on Kodak 4489 electron microscope film. Fourier transforms of 20 representative filaments in each case were computed and averaged using ImageJ (v1.34s, NIH, Bethesda, MD). All averaged transforms shown were brightness- and contrast-adjusted to show a clear signal; the adjustments for each condition were identical so that they could be objectively compared.

filament backbone (Fig. 1c), forming a compact but still helically disordered structure comparable to that seen at the earliest (10 ms) stage of activation.[16] Signs of axial reordering onto the 14.5-nm repeat were visible at this stage, as judged from the appearance of the Fourier transform (Fig. 1c). After 30 ms relaxation, the 48-nm layer line reappeared, demonstrating reordering of the heads onto the main (7,3) helical tracks[12,14] (Fig. 1d). At 50 ms, additional ordering became apparent, with the reappearance of the reflections at ~11.2 and ~9.8 nm (Fig. 1e), similar to the relaxed controls (Fig. 1a). Longer activation and relaxation times (hundreds of milliseconds) were also studied and gave similar results (data not shown).

Rapid removal of Ca^{2+} from briefly activated filaments simulates the process of relaxation following a twitch in live muscle. The reordering we observe occurs on the millisecond time scale, reaching a maximum well within the 100 ms taken for half-relaxation from peak tension in intact muscle[25] [while our experiments were carried out at a temperature 10 °C above the tension experiments, rapid reordering would be expected even at the lower temperature, assuming a Q10 of ~2 (Ref. 28)]. We conclude that reordering of the heads is a key physiological component of the relaxation process, rather than the result of slow annealing. The rate of reordering would be limited by the rate of Ca^{2+} loss from the Ca^{2+}-specific binding sites[29] on the essential light chains of the myosin heads. In the absence of nucleotide, this occurs with a half-time of ~13 ms.[27,30] Measurements in the presence of nucleotide have not been reported.

Intramolecular interaction between heads is a characteristic feature of the off-state of phosphorylation-regulated myosin molecules.[19,31,32] These interactions create an asymmetric structure in which the activity of both heads is switched off by the blocking of sites involved in actin-binding on one head and in ATP hydrolysis on the other. Similar head–head interactions are also present in the off-state of native, helically ordered myosin filaments (tarantula) regulated by phosphorylation.[18] In native filaments, additional interactions between the heads of axially neighboring myosin molecules along the helical tracks help to stabilize the off-state.[18] Similar head–head interactions have been demonstrated in Ca^{2+}-regulated (scallop[33]) myosin molecules and we have recently shown their presence in native scallop myosin filaments.[34] The rapidity with which these interactions are reestablished on Ca^{2+} removal from activated filaments (implied by the rapid helical reordering that we observe) suggests that their reformation is integral to the relaxation process of Ca^{2+}-regulated filaments. Our results suggest that this occurs in stages. The initial (within 20 ms) return of the heads to the filament backbone, forming a 'compact' structure with a degree of axial ordering (Fig. 1c), may result from reformation of the intramolecular head–head interactions. Following this, the heads may reestablish the main intermolecular interactions between adjacent axial levels along the 48-nm tracks, thus reforming the (7,3) helices.[12,14] The subsequent reappearance of the 11.2- and 9.8-nm reflections implies reformation of the (7,13) helical tracks and the axial perturbation.[14] Three-dimensional reconstruction of scallop filaments[34] suggests that the 11.2-nm reflection is due largely to the mass of the interacting motor domains lying along the (7,13) helical tracks. The return of this reflection might thus represent the final docking of the interacting head motor domains back onto the filament, possibly by binding to the associated S2.[18,31–33]

Effective relaxation is critical to normal muscle function. Reordering of heads near the filament backbone could contribute to the efficiency of this process by rapidly sequestering them away from the thin filaments (in addition to switching off their activity by the head–head and other interactions). This would be expected to minimize resistance to stretch that occurs when relaxed muscles return to their rest length. It may also reduce the work done by active fibers in shortening a muscle at low load in which unstimulated fibers are shortened passively. It could thus play an important role in the energetics and mechanics of relaxation.[17]

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