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Structural Changes Induced in Ca^{2+}-regulated Myosin Filaments by Ca^{2+} and ATP

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Abstract. We have used electron microscopy and proteolytic susceptibility to study the structural basis of myosin-linked regulation in synthetic filaments of scallop striated muscle myosin. Using papain as a probe of the structure of the head-rod junction, we find that this region of myosin is approximately five times more susceptible to proteolytic attack under activating (ATP/high Ca^{2+}) or rigor (no ATP) conditions than under relaxing conditions (ATP/low Ca^{2+}). A similar result was obtained with native myosin filaments in a crude homogenate of scallop muscle. Proteolytic susceptibility under conditions in which ADP or adenosine 5′-(β,γ-imidotriphosphate) (AMPPNP) replaced ATP was similar to that in the absence of nucleotide. Synthetic myosin filaments negatively stained under relaxing conditions showed a compact structure, in which the myosin cross-bridges were close to the filament backbone and well ordered, with a clear 14.5-nm axial repeat. Under activating or rigor conditions, the cross-bridges became clumped and disordered and frequently projected further from the filament backbone, as has been found with native filaments; when ADP or AMPPNP replaced ATP, the cross-bridges were also disordered. We conclude (*a*) that Ca^{2+} and ATP affect the affinity of the myosin cross-bridges for the filament backbone or for each other; (*b*) that the changes observed in the myosin filaments reflect a property of the myosin molecules alone, and are unlikely to be an artifact of negative staining; and (*c*) that the ordered structure occurs only in the relaxed state, requiring both the presence of hydrolyzed ATP on the myosin heads and the absence of Ca^{2+}.

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CONTRACTION of muscle is regulated via Ca^{2+}-dependent protein switches on the actin or myosin filaments or both, depending on the muscle and species. There are two basic forms of myosin-linked regulation. In one, found in vertebrate smooth and nonmuscle cells and in some invertebrate striated muscles, contraction is switched on by the Ca^{2+}-dependent phosphorylation of the myosin regulatory light chains (Sobieszek, 1977; Sherry et al., 1978; Adelstein and Eisenberg, 1980; Kendrick-Jones and Scholey, 1981; Sellers, 1981). In the other, exemplified by the scallop and occurring in many invertebrate striated muscles, contraction is initiated when Ca^{2+} binds directly to the myosin heads (Kendrick-Jones et al., 1970; Szent-Györgyi et al., 1973; Lehman and Szent-Györgyi, 1975).

The structural basis of myosin-linked regulation is not yet understood. In the case of the scallop, it has been established that the regulatory and essential light chains and the heavy chain are all involved in regulation (Szent-Györgyi et al., 1973; Collins et al., 1986; Vibert and Cohen, 1988), forming a regulatory domain in the neck region of the myosin head (Szentkiralyi, 1984; Winkelmann et al., 1984; Bennett et al., 1984). Fluorescence measurements give evidence for local structural changes in the myosin heads when they bind Ca^{2+} (Wells et al., 1985) or ATP, and there is evidence that these changes involve movement of the light chains (Hardwicke et al., 1983; Hardwicke and Szent-Györgyi, 1985; Wells et al., 1985). Large changes in the morphology of myosin molecules from vertebrate smooth muscle have been associated with the state of phosphorylation of the regulatory light chains (Craig et al., 1983; Trybus and Lowey, 1984; Ikebe and Hartshorne, 1984; Suzuki et al., 1985, 1988), and there is evidence that related changes may also occur in scallop myosin (Frado, L.-L. Y., and R. Craig, manuscript in preparation).

Evidence for regulatory changes in filament structure in both phosphorylation- and direct Ca^{2+}-dependent, myosin-regulatory systems has come from ultrastructural studies. In the switched off (relaxed) state, myosin filaments of both types have a regular, ordered structure that becomes disordered when the filaments are activated (Ikebe and Ogihara, 1982; Vibert and Craig, 1985; Craig et al., 1987). However, the basis of this order–disorder transition is not understood. It remains possible that the observed changes could be artifacts of the negative-staining procedure or may have been caused by interaction of myosin cross-bridges with actin or other proteins in the crude filament homogenates used in some cases. In this paper we show that neither of these possibilities is likely: first by comparing the results of native filaments with those of synthetic filaments made from purified myosin, where the possibility of interaction with actin is

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eliminated; and second by using susceptibility to proteolysis by papain as an independent test for the existence of structural differences between relaxed and activated filaments. The results support the earlier conclusions that activation causes a loosening of the binding of the myosin heads to the myosin filament backbone or to each other. This occurs independently of the presence of actin or other proteins and is accompanied by a change in structure or accessibility of the head-rod junction of scallop myosin.

Materials and Methods

Scallop Muscle

Scallops (*Aequipecten irradians*) were purchased from the Marine Biological Laboratories, Woods Hole, MA. Straited adductor muscles were harvested and stored at −20°C in buffer containing 20 mM NaCl, 0.5 mM $MgCl_2$, 0.05 mM EDTA, 1.5 mM NaN_3, 0.05 mM PMSF, 0.005% (wt/vol) sulfadiazine, 2.5 mM Na phosphate, pH 7.0, and 50% (vol/vol) ethylene glycol (glycolated muscle; Hardwicke et al., 1982).

Actin Preparation

An acetone powder of rabbit back and leg muscles was prepared using the method of Pardee and Spudich (1982). Actin was extracted from the acetone powder according to the method of Spudich and Watt (1971) and stored on ice.

Myosin Preparation

Scallop myosin was prepared according to the method of Chantler and Szent-Györgyi (1978) with the following modifications: (*a*) after mincing with scissors, tissue was sheared on ice with two 7-s bursts of a Polytron homogenizer (Brinkmann Instruments Co., Westbury, NY), at setting no. 5, with a 1-min intervening cooling period; and (*b*) myosin was collected from fractions precipitating between 45 and 55% $(NH_4)_2SO_4$. Myosin was stored on ice in high salt buffer (0.6 M NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 3 mM NaN_3, 20 mM Na phosphate) and used within 3 d of preparation.

Protein concentrations were determined using a method modified from that of Lowry et al. (1951) with BSA as a standard (Schacterle and Pollack, 1973). Myosin concentration was also estimated directly using an extinction coefficient $E^{1\%}_{280\ nm}$ of 5.3 cm^{-1} (Stafford et al., 1979).

Actin-activated myosin ATPase activities were assayed at 22°C in a 5-ml reaction mixture containing 20 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 30 mM Tris, pH 7.5, with or without 0.2 mM $CaCl_2$. 0.3 mg of 20 mg/ml stock myosin in high salt buffer containing Tris in place of phosphate was mixed for 5 min on ice with actin at a ratio (wt/wt) of 3:10 in the presence of 1.5 mM Mg-ATP and 0.4 M NaCl before transferring to the reaction mixture. The reaction was initiatied with the addition of 1 mM Mg-ATP and stopped with 1/3 vol of a solution containing 13.3% SDS and 0.12 M EDTA, pH 7.0. The phosphate released was assayed according to the colorimetric method of Taussky and Shorr (1953) with the modification described by White (1982).

Filament Preparation

"Relaxed" synthetic filaments were formed by dialyzing purified myosin in high salt against relaxing solution (0.1 M CH_3COONa, 3 mM $(CH_3COO)_2$-Mg, 1 mM EGTA, 1 mM NaN_3, 1 mM DTT, 2 mM Mg-ATP, 10 mM imidazole, pH 7.0) for 4 h or overnight at 4°C. To prepare rigor or activated synthetic filaments, the relaxed synthetic filaments were dialyzed overnight against rigor solution (relaxing solution without Mg-ATP), or activating solution (relaxing solution with 1.1 mM $CaCl_2$), respectively. Activated filaments were also often made from relaxed synthetic filaments by adding $CaCl_2$ directly to the relaxed preparations; filaments made in this way gave the same papain digestion and electron microscopy results as filaments prepared by dialysis. Rigor or activated filaments of equivalent quality and similar appearance were also made by directly dialyzing purified myosin in high salt against rigor or activating solutions. Judging by papain digestion and electron microscopy studies (see below), these filaments had similar structural properties to those produced by the two-stage dialysis method described above.

Native filaments were prepared from skinned, fresh, or from glycolated, (see section titled Scallop Muscle) scallop striated adductor muscle according to the method of Vibert and Craig (1983) with modifications. The minced muscle was homogenized twice on ice in buffer A (0.1 M NaCl, 8 mM $MgCl_2$, 5 mM ATP, 5 mM EGTA, 3 mM NaN_3, 1 mM DTT, and 10 mM Na phosphate, pH 7.0) for 1 s with a Polytron homogenizer at setting 5.5. The homogenate was spun at 2,000 rpm (SS34 rotor, Sorvall Instruments Div., Dupont Co., Newton, CT) at 4°C for 2 min to remove larger filaments and particles. The supernatant was spun at 8,000 rpm for 10 min and the pellet, containing thick and thin filaments, was resuspended in buffer A. The filament suspension was washed once with appropriate relaxing, rigor, or activating solution and resuspended in the same solution for papain digestion and electron microscopy.

Papain Digestion of Myosin Filaments

Before use, papain (Sigma Chemical Co., St Louis, MO) was incubated with 1 mM DTT, 5 mM Tris buffer, pH 7.5, at 35°C for 1 h as described by Ikebe and Hartshorne (1984). Synthetic or native myosin filaments were digested at 25°C at a myosin concentration of 3 mg/ml and a myosin to papain ratio (wt/wt) of 5 or 10:1 in relaxing, activating, or rigor solution (see Results). At prescribed intervals, digestion was stopped with 5 mM iodoacetic acid (Sigma Chemical Co.). Digested samples were treated with equal volumes of SDS–polyacrylamide gel sample buffer (Laemmli, 1970), and boiled for 3 min before gel electrophoresis.

Electron microscopic and papain digestion studies were also performed on filaments in relaxing solution in which Mg-ADP (0.5–2.0 mM) or Mg-adenosine 5′-(β,γ-imidotriphosphate) (Mg-AMPPNP)[1] (2 mM) (both Sigma Chemical Co.) replaced Mg-ATP. Nucleotide replacement was accomplished by dialyzing relaxed synthetic filaments against rigor conditions overnight (or by directly making synthetic filaments from myosin in rigor conditions), and then adding Mg-ADP or Mg-AMPPNP. To ensure complete removal of ATP from these solutions, an ATP depletion system containing 50 μg/ml hexokinase, 1 mM glucose, and 200 μM AP_5A (P^1,P^5-di(adenosine-5′) pentaphosphate) was used (Padrón and Huxley, 1984). Papain digestion and electron microscopic experiments were started from 5 to 30 min after the addition of nucleotides.

SDS-PAGE

Gel electrophoresis was carried out according to the method of Laemmli (1970), using standard sized 10–20% gradient gels (Integrated Separation Systems, Hydê Park, MA) which were run on a Pharmacia Fine Chemical Co. (Piscataway, NJ) electrophoresis apparatus. The gels were stained with 0.1% Coomassie brilliant blue R-250, and destained with a methanol–acetic acid solution for photography and scanning.

Gels were scanned and analyzed with an LKB Instruments, Inc. (Gaithersburg, MD) Ultroscan XL scanner. Relative heavy chain areas were plotted against time in order to compare rates of heavy chain digestion.

The molecular mass of proteolytic fragments in kilodaltons was determined by comparison with those of standard proteins (Sigma Chemical Co.) run on the same gel.

Electron Microscopy

Myosin filaments were applied to grids coated with freshly prepared thin carbon films; the grids were then rinsed with the appropriate solution (relaxing, rigor, or activating, etc.) and negatively stained with 3% uranyl acetate. The filament solutions had acetate as the major anion (see Filament Preparation section above) since, under relaxing conditions, this preserved the appearance of the ordered and compact synthetic filaments best. Washing with relaxing solution in which chloride and/or phosphate replaced acetate produced much less distinct images, even though papain digestion of synthetic filaments made with chloride and/or phosphate buffers was the same as that of filaments made with acetate buffer (data not shown).

To study papain digestion products, papain-digested filaments were dissolved in 0.5 M CH_3COONH_4 in a 55% (vol/vol) glycerol/water mixture and the solution sprayed on to freshly cleaved mica; specimens were rotary shadowed with platinum and carbon coated in an Edwards High Vacuum, Inc. (model E306A; Grand Island, NY) coating system (Craig et al., 1983). Replicas were floated off the mica and picked up with 400-mesh grids. Samples were examined in a JEOL USA (Peabody, MA) 100CX electron micro-

1. *Abbreviations used in this paper*: AMPPNP, adenosine 5′-(β,γ-imidotriphosphate); HMM, heavy meromyosin; LMM, light meromyosin; S1, subfragment 1 of myosin.

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scope operated at 80 kV with an anticontamination device. Grids were calibrated using the 39.5-nm repeat of tropomyosin paracrystals (Caspar et al., 1969) recorded at the same magnification as the experimental pictures.

Optical Diffraction

Optical diffraction patterns of electron micrographs were recorded on Panatomic-X film (Eastman Kodak Co., Rochester, NY) using a diffractometer built to the design of Salmon and DeRosier (1981).

Results

Characterization of Myosin

Scallop striated adductor myosin was 98% pure and had no detectable actin, paramyosin, or tropomyosin contamination as judged from SDS–acrylamide gel analysis (Figs. 1, 3, and 4, *M*). The myosin had actin-activated Mg-ATPase activity of 400–900 nmol/mg per min in the presence of Ca^{2+} and 20–40 nmol/mg per min in the absence of Ca^{2+}. The calcium sensitivity [(1 − rate in absence of Ca^{2+}/rate in presence of Ca^{2+}) × 100] (Chantler and Szent-Györgyi, 1978) was >93%.

Proteolytic Susceptibility of Synthetic Myosin Filaments

With limited time of digestion, papain preferentially attacks myosin at the head-rod junction, with slower attack at a site on the head and at the light meromyosin (LMM) and heavy meromyosin (HMM) junction (Lowey et al., 1969; Stafford et al., 1979; Craig et al., 1980; Onishi and Watanabe, 1984; Ikebe and Hartshorne, 1986). Therefore, we have used susceptibility to papain digestion as a probe of the structure and/or accessibility of the head-rod junction under different experimental conditions.

In the presence of ATP and the absence of Ca^{2+} ($<10^{-8}$ M) (relaxing solution), papain treatment of synthetic filaments caused very slow digestion of the 205-kD myosin heavy chain (Figs. 1 *a*, 2, and 3 *a*). The initial products were 175-, 150-, and 135-kD fragments and were themselves resistant to further digestion (Figs. 1 *a* and 3 *a*). There was no band running at the molecular mass of the subfragment 1 (S1) heavy chain (97 kD). Most of the myosin, therefore, was resistant to papain degradation during the first 5 min of the digestion period. To aid identification of fragments, digested speci-

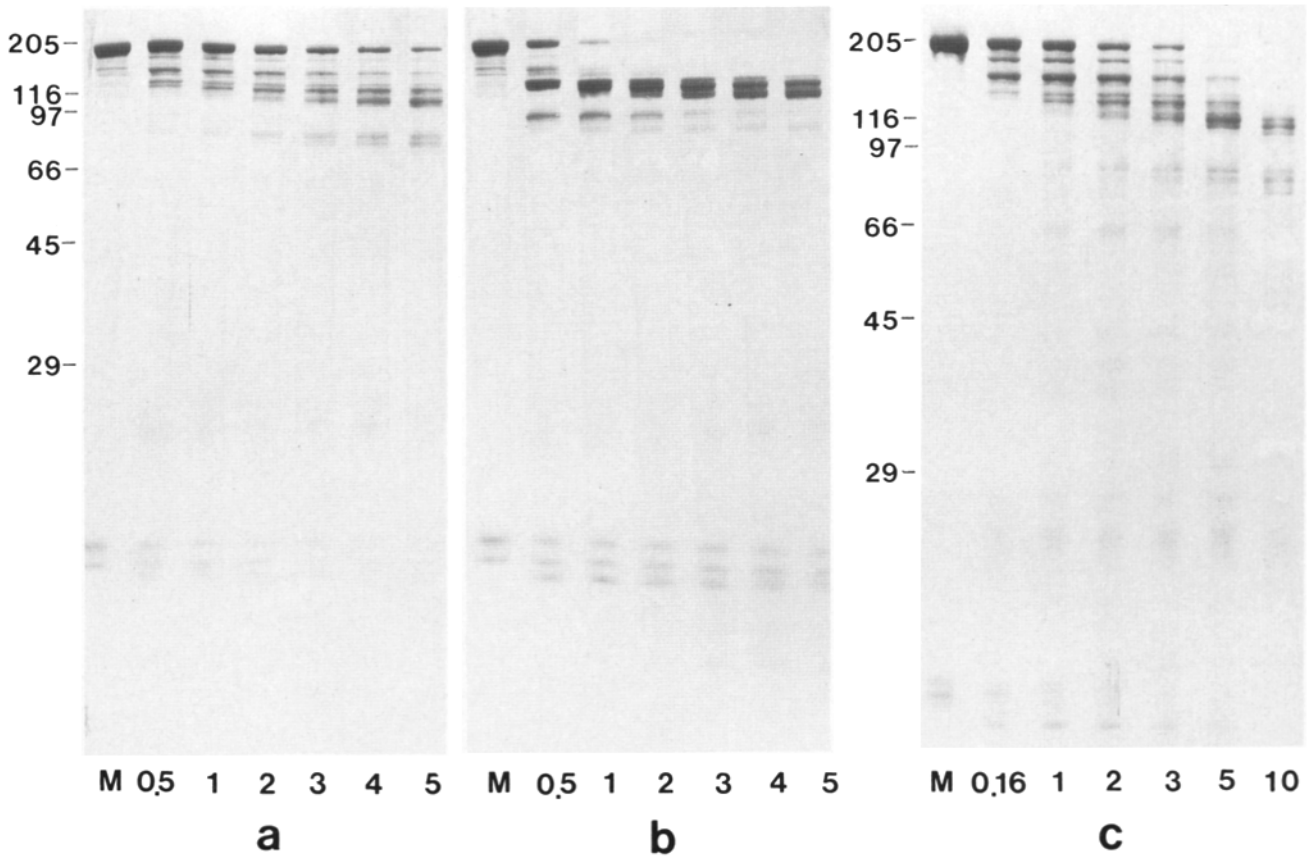


Figure 1. SDS-PAGE (10–20% gradient gel) of papain-digested synthetic myosin filaments showing (*a*) slow digestion of 205-kD heavy chains in relaxing solution; (*b*) fast digestion in activating solution; and (*c*) slow digestion of re-relaxed filaments (additional 2 mM EGTA added after the incubation of filaments in activating solution). Synthetic filaments in 0.1 M CH_3COONa, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3, 1 mM DTT, 2 mM Mg-ATP, 10 mM imidazole, pH 7.0, were digested with papain at 25°C for various lengths of time (*bottom*, given in minutes) at a myosin to papain ratio (wt/wt) of 10:1 in the presence (*b*) or absence (*a*) of 0.1 mM free Ca^{2+}, and a ratio of 5:1 in the sample with Ca^{2+} plus additional EGTA (*c*). Note that even when twice the normal amount of papain was used, the heavy chains were digested more slowly with EGTA and Ca^{2+} (*c*) than with just Ca^{2+} (*b*). The molecular mass standards in kilodaltons are indicated on the left of *a* and *c*. *M*, Myosin.

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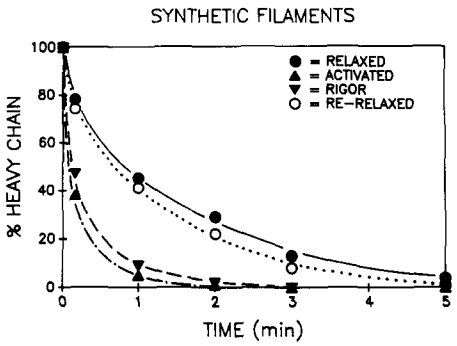


Figure 2. Plot of the percentage of heavy chain vs. time during digestion of synthetic filaments by papain in ATP/low Ca^{2+} (●), ATP/high Ca^{2+} (▲), ATP/high Ca^{2+} with additional 2 mM EGTA (○), and no ATP/low Ca^{2+} (▼) (See Fig. 1). Digestion was carried out at myosin/papain weight ratio of 5:1.

mens treated with iodoacetic acid were dissolved in high salt and rotary shadowed. During early digestion times (up to 3 min), the digested samples contained mostly intact myosin together with a mixture of HMM, LMM, rod, and myosin molecules with the wide part of the heads missing. S1 was rarely seen at any of the time points. The digested fragments at 175 and 150 kD are HMM (Szent-Györgyi et al., 1973; Shpetner, 1985; Frado, L.-L. Y., and R. Craig, manuscript in preparation). The smaller fragments at 135, 116, and 80 kD are probably myosin with partially digested heads and fragmented rods. It thus appears that in the presence of ATP and absence of Ca^{2+}, the head-rod junction is protected from proteolytic attack and that the most susceptible region is the LMM/HMM junction.

In the presence of ATP and high free Ca^{2+} (0.1 mM) (activating solution) or in the absence of both (rigor solution), myosin heavy chain was nearly completely digested after a 1-min incubation with papain (Figs. 1 *b* and 3 *b*). The predominant fragments of 125 and 110 kD correspond to the molecular mass of the rod (Margossian et al., 1981; Okamoto and Sekine, 1987). There was also a population of fragments, not present in the digest of relaxed filaments, at 97 kD corresponding to the molecular mass of S1 (Szent-Györgyi et al., 1973; Craig et al., 1980: Shpetner, 1985). In rotary-shadowed digests treated with high salt, S1 and rod were the predominant fragments seen (not shown). These results suggest that in the presence of ATP and Ca^{2+}, or in the absence of ATP, the head-rod junction is highly susceptible to papain digestion.

The effects of Ca^{2+} on the digestion pattern and rate were

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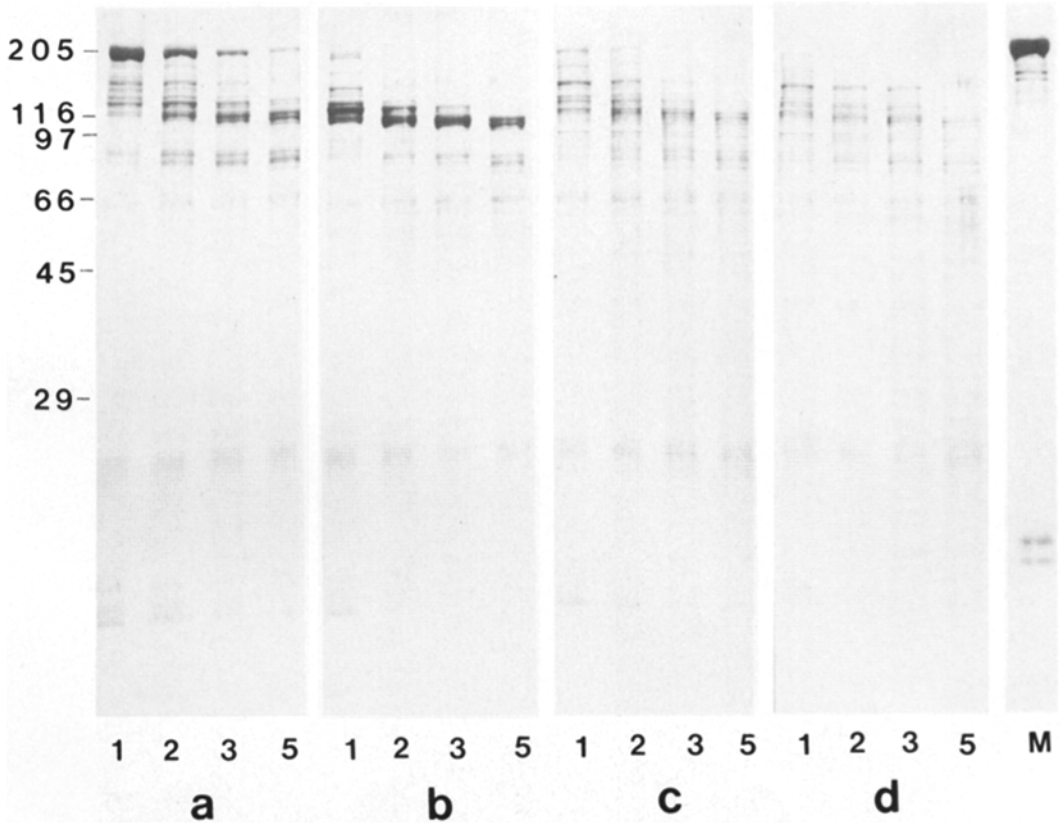


Figure 3. SDS-PAGE (10–20% gradient gel) showing the digestion products of synthetic filaments in (*a*) the absence of Ca^{2+} and the presence of ATP (relaxing solution); (*b*) the absence of ATP (rigor solution); (*c*) the presence of ADP (relaxing solution with 2 mM Mg-ADP replacing Mg-ATP and with the ATP-depletion system); and (*d*) the presence of AMPPNP (relaxing solution with 2 mM Mg-AMPPNP replacing Mg-ATP). Digestion was carried out at a myosin to papain (wt/wt) ratio of 5:1 causing the S1 band to be weak even at early digestion times (cf. Fig. 1 *b*). In the absence of ATP, heavy chains were rapidly digested. ADP and AMPPNP could not substitute for ATP in retarding the rate of heavy chain digestion. Duration of digestion time is indicated in minutes on the bottom. The molecular mass in kilodaltons is indicated on the left. *M*, myosin.

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reversible. When EGTA was added to remove Ca^{2+} from the activated filaments, digestion of the myosin heavy chain slowed again, and no obvious S1 and rod fragments were produced (Figs. 1 *c* and 2).

The rate of digestion of the myosin heavy chain in activated filaments is about four to five times that in relaxed filaments (Fig. 2). The rate of digestion in rigor filaments is similar to, but slightly slower than, that in activated filaments (Fig. 2). When ADP replaced ATP in the relaxing solution, and an ATP-depletion system was present to remove trace ATP (see Materials and Methods), the digestion of myosin heavy chain was rapid and similar to the rate of digestion of filaments in the absence of ATP and Ca^{2+} (Fig. 3, *b* and *c*). However, when the ATP-depletion system was not used, the digestion rate was as slow as that under relaxing conditions (data not shown). When AMPPNP was used instead of ATP, with or without the ATP-depletion system, the degradation of myosin heavy chain was rapid in the absence of Ca^{2+}, and again similar to the rate of digestion of filaments in the absence of both ATP and Ca^{2+} (Fig. 3, *b* and *d*). The digestion patterns observed under rigor, ADP plus the ATP-depletion system, and AMPPNP conditions were similar to those of activated filaments.

Proteolytic Susceptibility of Native Myosin Filaments

Native filament homogenates from either fresh or glycolated muscle contain actin, paramyosin, tropomyosin, and small amounts of other proteins in addition to myosin (Fig. 4, *M* and *C*; Szent-Györgyi et al., 1973). However, rates and patterns of digestion of the myosin heavy chain of native filaments in relaxed, activated, and rigor conditions are essentially unaffected by the presence of these other components and are similar to those of the synthetic filaments (compare Figs. 1, 3, and 4). The rates and patterns of digestion of native filaments isolated from fresh muscle were the same as those of filaments isolated from glycolated muscle. In relaxing solution, papain digestion of the myosin heavy chain was at least five times slower than that of filaments in activating or rigor solutions (Figs. 4 and 5). Native filaments incubated in the same conditions, but without papain, served as controls. Since no digestion of the filament proteins was observed in any of the controls, the increased rate of digestion observed in the presence of high Ca^{2+} was not due to Ca^{2+}-activated proteases. As with synthetic filaments, digestion in rigor solution appeared to be slightly but reproducibly slower than in activating solution (Fig. 5).

Electron Microscopy of Synthetic Myosin Filaments

Synthetic filaments under different biochemical conditions were examined by negative staining. Filaments made by dialyzing against relaxing solution ranged up to at least 3.5 μm in length (longer than isolated native filaments) and had di-

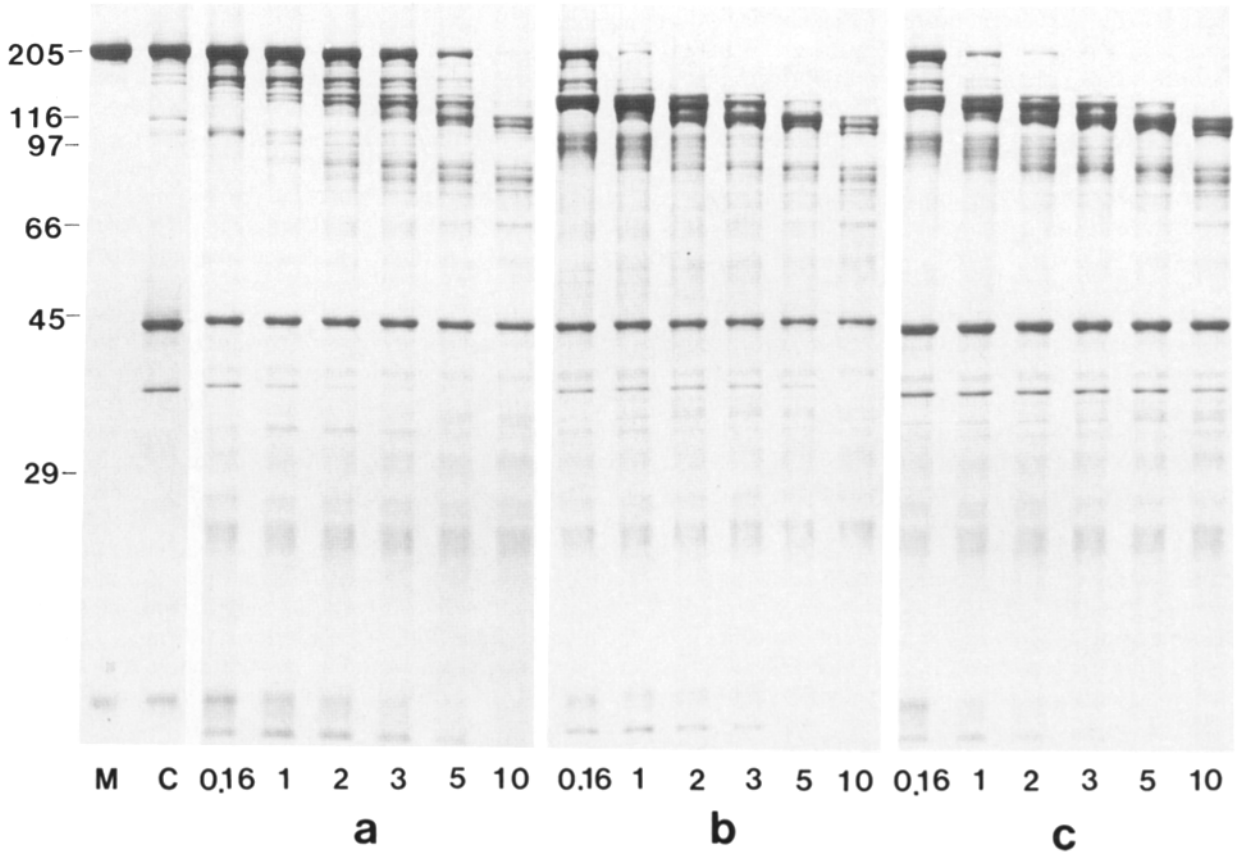


Figure 4. SDS-PAGE (10–20% gradient gel) of papain-digested native filaments in crude filament homogenate showing (*a*) slow digestion of myosin heavy chain in relaxing solution (ATP/low Ca^{2+}); (*b*) fast digestion in activating solution (ATP/high Ca^{2+}); and (*c*) fast digestion in rigor solution (no ATP/low Ca^{2+}). Digestion was carried out at a myosin to papain (wt/wt) ratio of 10:1. Duration of digestion time is indicated in minutes on the bottom. The molecular mass in kilodaltons is indicated on the left. *M*, Myosin; *C*, control native filaments before digestion.

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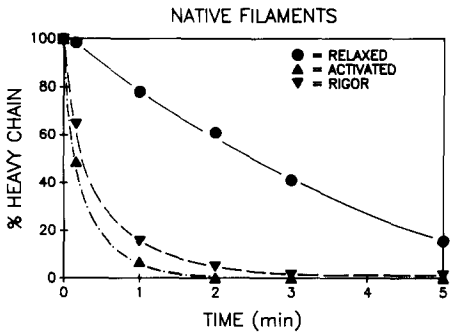


Figure 5. Plot of the percentage of heavy chain vs. time during digestion of native filaments by papain in ATP/low Ca^{2+} (relaxing solution; ●), ATP/high Ca^{2+} (activating solution; ▲), and no ATP/low Ca^{2+} (rigor solution; ▼). Digestion was carried out at a myosin/papain weight ratio of 10:1.

ameters (measured between the outer edges of the cross-bridges) averaging 41 ± 3 nm (mean ± SD). Their structure was compact (i.e., the cross-bridges were close to the filament backbone) and they showed a regular, ordered appearance with a 14.5-nm periodicity similar to that seen in native filaments (Fig. 6, *a* and *b*). Occasional signs of helical tracks of cross-bridges were observed (cf. Vibert and Craig, 1983), although these were not very clear. Optical diffraction patterns confirmed the presence of the 14.5-nm repeat but did not show any off-meridional layer lines (Fig. 6 *b*, *inset*).

When synthetic filaments were treated with activating solution either before or after placing them on grids, they showed disordering and clumping of the cross-bridges and had diameters averaging 46 ± 7 nm (Fig. 6, *c* and *d*). This change from ordered to disordered structure, also observed in native filaments, cannot be due to interaction with actin, since actin is absent from synthetic filaments. The disordered structure in activated filaments reverted to an ordered, compact structure with a 14.5-nm repeat when Ca^{2+} was removed via the addition of EGTA (Fig. 6, *e* and *f*).

In synthetic filaments dialyzed against rigor solution the cross-bridges were clumped and disordered, but apparently not as strongly as in the activated state (Fig. 7, *a* and *b*); disorder was also induced by placing relaxed (ordered) filaments on a grid and rinsing extensively (20 min) with rigor solution to remove ATP. When ADP (without the ATP-depletion system) replaced ATP in relaxing solution, the synthetic filaments looked as ordered as the filaments in relaxing solution (data not shown). This ordered structure was observed in filaments with an ADP concentration as low as 50 μM. However, when the ATP-depletion system was used with ADP, synthetic filaments appeared disordered (Fig. 7, *c* and *d*). In the presence of AMPPNP (with or without the ATP-depletion system) instead of ATP, synthetic filaments were disordered (Fig. 7, *e* and *f*).

Discussion

Using electron microscopy, we have demonstrated that relaxed synthetic scallop myosin filaments change from an ordered to a disordered arrangement of cross-bridges when activated by calcium; similar changes are also reported by Vibert and Castellani (1989) in the accompanying paper. To investigate these effects further, we have used susceptibility to papain digestion as an independent probe of myosin structure. The differences we observe in rates and sites of papain attack on myosin demonstrate that molecular conformation or accessibility changes on activation, supporting the view that the structural changes seen with the electron microscope are real and not artifacts of negative staining. They further suggest that one site of the structural change we observe is the head–tail junction, the preferred site of attack by papain. Both papain digestion and ultrastructural studies demonstrate that the Ca^{2+} effects are reversible, providing further evidence that the changes are not artifactual. Our findings support earlier electron microscopic observations of an order–disorder transition occurring on activation of native filaments of scallop (Vibert and Craig, 1985) and tarantula striated muscle (Craig et al., 1987). We also find changes in proteolytic susceptibility of native filaments that are similar to those in synthetic filaments. Thus, synthetic filaments provide a good model system for studying structural changes in relation to activation, and, indeed, are superior to native filaments in several ways. For example, the results from synthetic filaments show directly that the disorder previously observed in native filaments is unlikely to have been a result of cross-bridge interaction with actin or other proteins (e.g., paramyosin) in the crude filament preparations used, since the synthetic filaments are made from purified myosin. Synthetic filaments also provide an important link in comparing structural transitions in native filaments with those of isolated myosin molecules (Frado, L.-L. Y., and R. Craig, manuscript in preparation).

It is possible that the conformational changes that cause the differences in papain susceptibility between relaxed and activated filaments are exactly those ones we have observed by electron microscopy. In the activated state the loose association of the myosin heads with the filament backbone (suggested by the disordering of the cross-bridges) would appear to make the head–tail junction readily accessible to papain: this is consistent with the finding that papain digestion of activated filaments yielded a predominant degradative product of 97 kD that, under the electron microscope, appeared to consist mainly of myosin heads (S1). In the relaxed state the head–tail junction appears to be highly protected from papain digestion, consistent with the apparently tight association of the heads with the backbone (suggested by the ordered and compact arrangement of cross-bridges). However, other conformational effects must also contribute to the change in papain susceptibility since Ca^{2+}-dependent changes in digestibility of the head–tail junction have also been observed with HMM, a soluble fragment of myosin; there is some correlation of these changes with the angle of the myosin heads with respect to the tail (Frado, L.-L. Y., and R. Craig, manuscript in preparation; Suzuki et al., 1985, 1988). It thus seems likely that the release of myosin heads from the backbone on activation may be a result of the change in the angle of the heads, or both may be a result of some more subtle change in the heads (cf. Ikebe and Hartshorne, 1984; Hardwicke et al., 1983; Hardwicke and Szent-Györgyi, 1985).

The 14.5-nm repeat in the relaxed scallop synthetic myosin filaments is similar to that in native scallop filaments (Vibert and Craig, 1983) and is much more regular than that in synthetic filaments formed from other (mainly rabbit) striated muscle myosins (e.g., Huxley, 1963; Moos et al., 1975;

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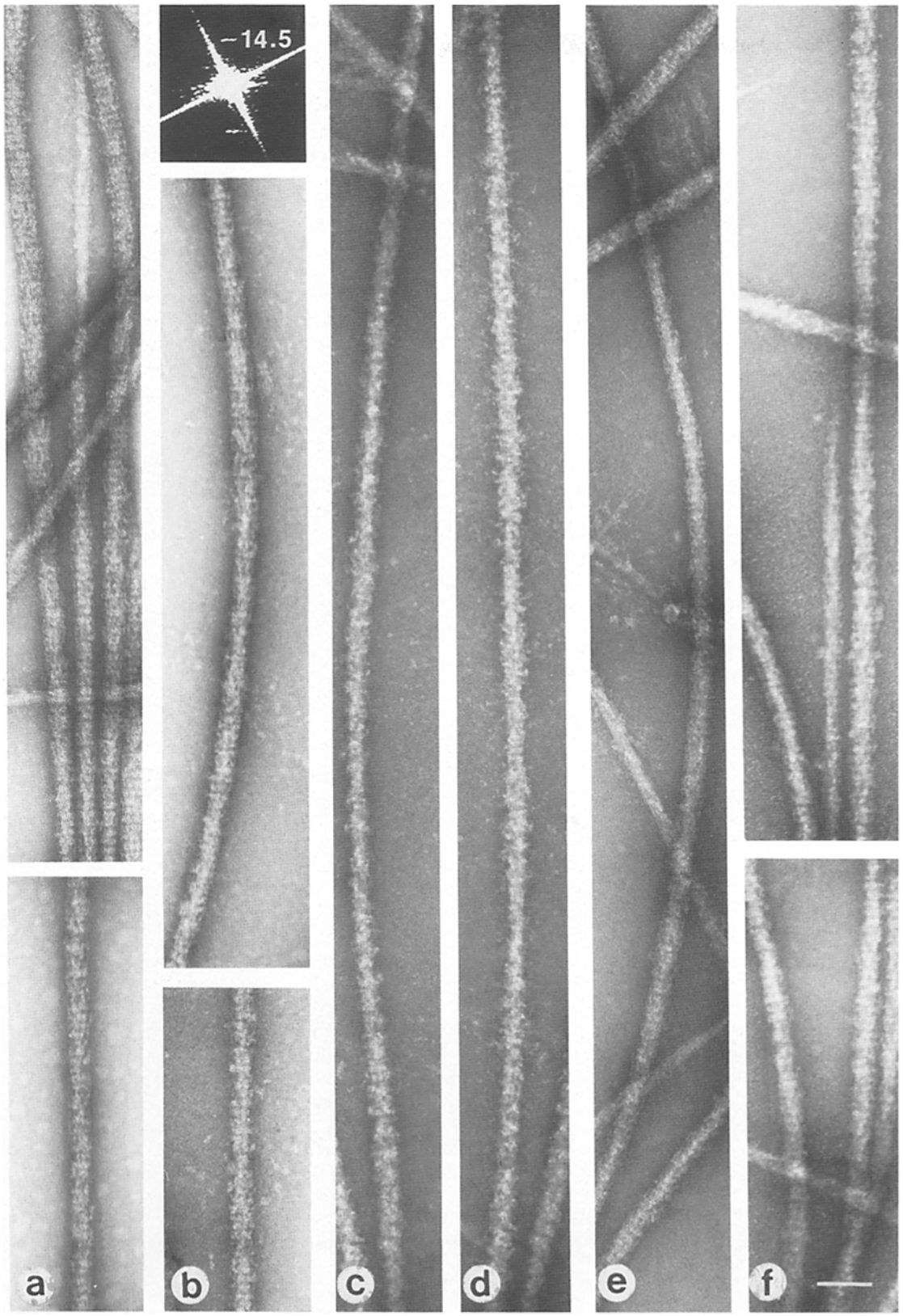


Figure 6. Electron micrographs of negatively stained synthetic myosin filaments (*a* and *b*) in relaxing conditions (ATP/low Ca^{2+}) showing ordered structure; (*c* and *d*) in activating conditions (ATP/high Ca^{2+}) showing disordered and clumped structure; and (*e* and *f*) in relaxing conditions after activating conditions (addition of further EGTA to bind free Ca^{2+}) showing ordered structure. (*b*, *top inset*) Optical diffraction pattern of relaxed synthetic filaments showing 14.5-nm meridional reflection. Bar, 100 nm.

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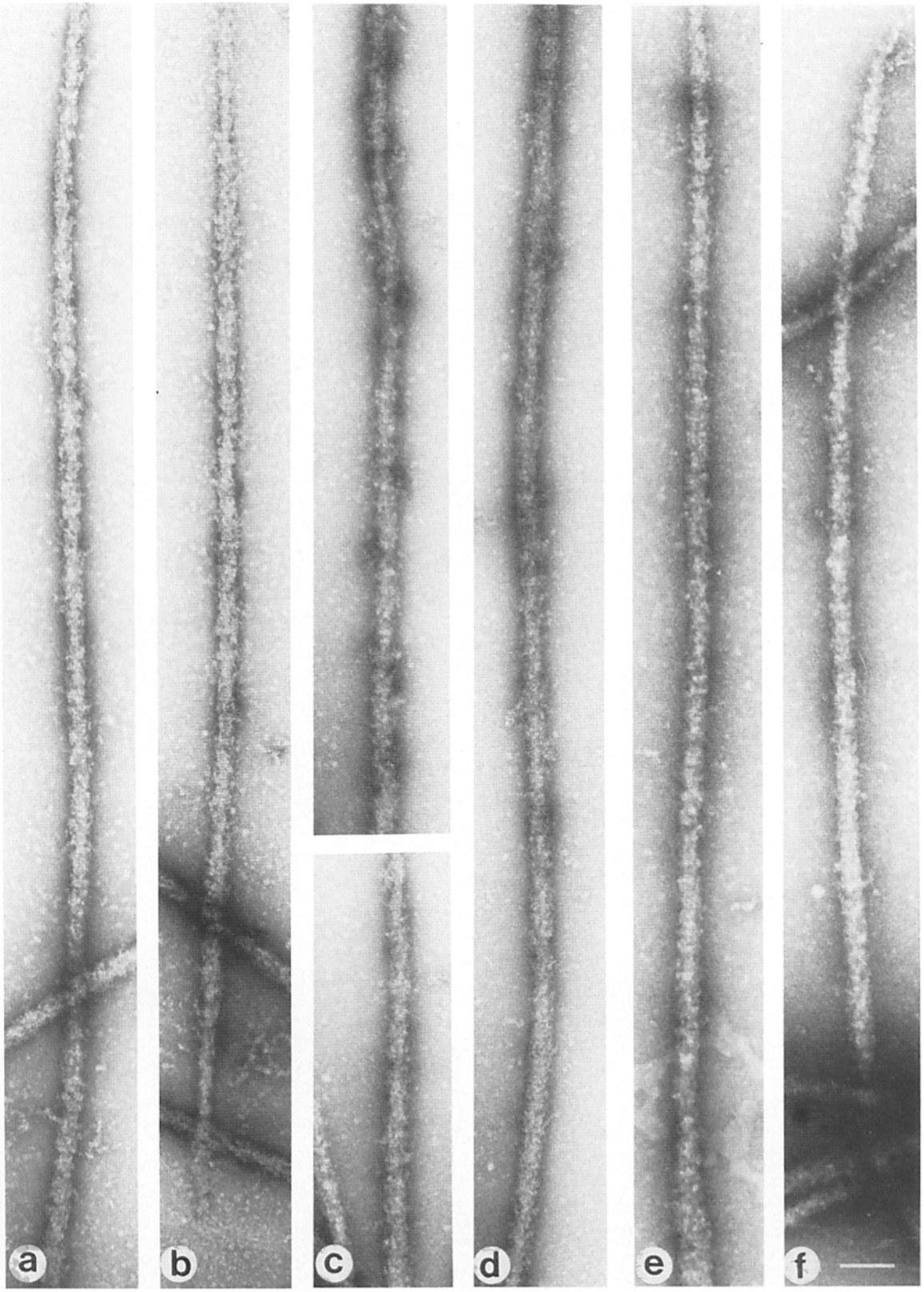


Figure 7. Electron micrographs of negatively stained synthetic myosin filaments (*a* and *b*) in rigor solution (no nucleotide/low Ca^{2+}) showing clumped/disordered structure; (*c* and *d*) in relaxing solution with 2 mM Mg-ADP replacing Mg-ATP and with the ATP-depletion system (ADP/low Ca^{2+}) showing clumped/disordered structure but with hint of order in some areas; (*e* and *f*) in relaxing solution with 2 mM Mg-AMPPNP replacing Mg-ATP (AMPPNP/low Ca^{2+}) showing clumped/disordered structure. Bar, 100 nm.

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Pollard, 1975; Hinssen et al., 1978; Pinset-Härström and Truffy, 1979; Pinset-Härström, 1985). This may be related to the fact that some of these other studies were done in the absence of ATP, conditions under which we find a disordered structure, and/or it may reflect a generally greater stability of the myosin cross-bridge array in regulated myosin filaments (cf. Hinssen et al., 1978; Ikebe and Ogihara, 1982; Kensler and Levine, 1982; Craig, R., unpublished data), which can be switched off under relaxing conditions (giving rise to an ordered structure), compared with unregulated myosins (e.g., rabbit) which cannot be switched off. In the relaxed state the myosin heads appear to be closely associated with the filament backbone. However, the precise arrangment of the two heads within each cross-bridge is uncertain. Three-dimensional reconstruction of native scallop filaments from negatively stained images were most simply interpreted in terms of two heads running parallel to each other and pointing away from the myosin tail (Vibert and Craig, 1983). On the other hand, rotary shadowing of scallop HMM suggests that the heads tend to point back toward the tail in relaxing conditions (Frado L.-L. Y., and R. Craig, manuscript in preparation), while more detailed reconstructions from other species of myosin filament (Crowther et al., 1985; Levine et al., 1988) suggest that one head points up the filament and the other points down.

The presence of ATP and absence of Ca^{2+} (i.e., relaxing conditions) both appear to be required for the ordered, compact structure. This conclusion follows from comparison of results obtained with and without ATP and with ATP analogues. Synthetic filaments in the rigor state, at low as well as high Ca^{2+}, appeared disordered, as found in native filaments (e.g., Vibert and Craig, 1985; Clarke et al., 1986), although the disorder did not appear to be quite as great as that in activated filaments. This correlated with a high rate of papain digestion of heavy chains of rigor filaments similar to, although not quite as fast as, that of activated filaments. The small amount of order appears unlikely to be due to trace ATP remaining in the rigor solution since, even in the presence of the ATP-depletion system, rigor filaments still looked similar to those without the depletion system, and the rates of papain digestion of heavy chains were the same. Replacing ATP with ADP in the absence of Ca^{2+} did not produce an ordered structure, and the rate of papain digestion was high, provided that care was taken to eliminate all ATP by means of the ATP-depletion system. If the ATP-depletion system was not used, ordered filaments were observed (and the rate of digestion was slow), presumably due to the presence of low levels of ATP. While these results could be explained on the basis of ATP contamination of ADP, this seems unlikely since the ordered structure was observed at ADP concentrations as low as 50 μM. It seems more likely that traces of myokinase were active in the myosin preparation, but that in the presence of the ATP-depletion system, ATP production was slowed and any trace ATP produced was removed. When the ATP analogue AMPPNP, which is not hydrolyzed, replaced ATP in the relaxing solution, ordered structures were not obtained and the rate of papain digestion was high. Thus, by the criteria of cross-bridge order or papain susceptibility, neither ADP nor AMPPNP can substitute for ATP in the relaxing solution. We conclude that hydrolyzed ATP (ADP.Pi) is required for the ordered structure occurring in the relaxed state, consistent with the finding of Wells and Bagshaw (1985) that relaxed scallop myosin is in the state M.ADP.Pi, where the products of ATP hydrolysis are trapped on the myosin heads.

In solution, regulation of actin-activated scallop myosin ATPase activity occurs mainly through inhibition of a kinetic step of the ATPase cycle happening after the heads have bound to actin, rather than by inhibition of binding of the heads to actin per se (Chalovich et al., 1984). In the intact filament lattice, an additional level of control may be present which is not detectable in solution: the tight association of the myosin heads with the backbone in the relaxed state (suggested by the ordered array of cross-bridges) may contribute to the low level of actin-activated myosin ATPase in relaxed muscle by restricting the binding of myosin heads to actin. Loosening of the heads from the backbone on activation (suggested by the disordering of the heads on binding of Ca^{2+}) may facilitate actin–myosin interaction in contracting muscle (Vibert and Craig, 1985; Craig et al., 1987). Wells and Bagshaw (1985) have shown that scallop myosin ATPase activity is also regulated by Ca^{2+} even in the absence of actin. It is therefore also possible that the disorder we observe is a reflection or a result of a change in conformation of myosin from a structure that turns ATP over slowly to one in which it can turn ATP over more rapidly. We are currently carrying out experiments using the rapid freezing technique (Heuser et al., 1979; Padrón et al., 1988) to test whether the structural changes described in this paper occur in intact scallop muscle in the rigor and activated states.

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