Direct Localization of Monoclonal Antibody-binding Sites on *Acanthamoeba* Myosin-II and Inhibition of Filament Formation by Antibodies That Bind to Specific Sites on the Myosin-II Tail

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ABSTRACT Electron microscopy of myosin-II molecules and filaments reacted with monoclonal antibodies demonstrates directly where the antibodies bind and shows that certain antibodies can inhibit the polymerization of myosin-II into filaments. The binding sites of seven of 23 different monoclonal antibodies were localized by platinum shadowing of myosin monomer-antibody complexes. The antibodies bind to a variety of sites on the myosin-II molecule, including the heads, the proximal end of the tail near the junction of the heads and tail, and the tip of the tail. The binding sites of eight of the 23 antibodies were also localized on myosin filaments by negative staining. Antibodies that bind to either the myosin heads or to the proximal end of the tail decorate the ends of the bipolar filaments. Some of the antibodies that bind to the tip of the myosin-II tail decorate the bare zone of the myosin-II thin filament with 14-nm periodicity. By combining the data from these electron microscope studies and the peptide mapping and competitive binding studies we have established the binding sites of 16 of 23 monoclonal antibodies. Two of the 23 antibodies block the formation of myosin-II filaments and given sufficient time, disassemble preformed myosin-II filaments. Both antibodies bind near one another at the tip of the myosin-II tail and are those that decorate the bare zone of preformed bipolar filaments with 14-nm periodicity. None of the other antibodies affect myosin filament formation, including one that binds to another site near the tip of the myosin-II tail. This demonstrates that antibodies can inhibit polymerization of myosin-II, but only when they bind to key sites on the tail of the molecule.

Acanthamoeba myosin-II has structural and enzymatic properties that are thought to be essential for its presumed role in cell motility (16, 20). Myosin-II has two heads and a long tail and consists of two heavy chains (~175,000 mol wt) and four light chains (two each at ~17,500 and ~16,500 mol wt) that yield a native molecular weight of ~400,000 mol wt. Each head is made up of part of a heavy chain and two light chains. The tail is made up of the carboxy-terminal ends of the heavy chains, presumably (by analogy to other myosins) arranged in alpha-helical coiled coils. In low salt, myosin-II molecules aggregate laterally to form small bipolar filaments similar to the larger bipolar filaments formed by other cytoplasmic myosins and by muscle myosins (18). The myosin-II heads contain the ATP- (17) and actin-binding sites (20). By analogy to muscle, the heads are believed to have the enzymatic activity responsible for mechanochemical energy transduction. The aggregation of tails to form bipolar filaments presumably allows myosin-II molecules to act in a polarized and concerted fashion during their interaction with actin to produce force.

It has not yet been possible to cleave myosin-II with proteases into distinct functional domains like muscle myosin subfragment-1, heavy meromyosin, subfragment-2, or light meromyosin (3). For example, brief digestion with trypsin cleaves the myosin-II heavy chain at a single site somewhere in the head but the two resulting polypeptides (70,000 and

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105,000 mol wt) can only be separated under denaturing conditions. As a result, the functional properties of the various structural domains cannot be discerned by a strictly biochemical approach.

To sort out the functions of the various parts of myosin-II, we prepared and characterized 23 monoclonal antibodies against the myosin-II heavy chain (10). These antibodies recognize at least 15 unique antigenic determinants and can be grouped into six families whose members are related by the proximity of their binding sites on the myosin-II heavy chain. They offer a unique opportunity to determine the function of each domain within the context of the whole myosin molecule.

Here we localize the binding site of four of the six antibody families on myosin-II and evaluate the ability of each antibody to block myosin-II filament formation. Antibody-binding sites on the heads, on the proximal part of the tail near its junction with the heads, and at the tip of the tail were all localized on individual myosin molecules by electron microscopy of platinum-shadowed antibody-myosin complexes. Antibodies were localized on myosin-II filaments reacted with antibody by electron microscopy of negatively stained preparations. By electron microscopy, we also demonstrate that two of the 23 antibodies inhibit the formation of myosin-II filaments and can slowly disassemble preformed filaments. As might be expected, these antibodies belong to the family that binds to the tip of the myosin-II tail. Antibodies that bind to the tail close to the heads do not prevent polymerization. The structural information that these studies provide are essential for the interpretation of antibody inhibition of myosin-II ATPase activity and contraction of gels of amoeba cytoplasm (11, 12).

Preliminary accounts of this work were presented at meetings of the American Society for Cell Biology and the Biophysical Society (7–9).

MATERIALS AND METHODS

Myosin-II purification, the production and purification of monoclonal antibodies, and the sources of most reagent grade chemicals were described previously (10). Glycerol and ammonium formate were obtained from J. T. Baker (Phillipsburg, NJ).

Each antibody is named according to its eliciting antigen (M2 for myosin-II), followed by a unique integer suffix that designates the individual clone that produces it (10).

Platinum Shadowing of Antibody-Myosin Complexes: The antibody-binding sites on the myosin-II molecule were determined by rotary shadowing antibody-myosin complexes dried from glycerol (5, 6, 22). Myosin-II (0.5–3.5 μM in 0.6 M ammonium formate, pH 7.5) was mixed with a small volume (typically 6% or less of the total volume of the mixture, to minimize sucrose concentration in the final sample) of antibody in 20% sucrose, 5 mM imidazole, pH 7.0, 3 mM NaN_3. The molar ratio of antibody to myosin varied from 1 to 10. The samples were diluted with ammonium formate to final myosin concentrations of 0.05 to 0.5 μM and mixed 1:1 with glycerol at room temperature. Samples were immediately sprayed onto freshly cleaved mica with an Effa Spray Mounter (Ernest F. Fullam, Inc., Schenectady, NY) and dried under vacuum to $1–10 \times 10^{-7}$ torr at room temperature for ~60 min. The time between dilution of samples into glycerol and spraying was typically <3 min (see reference 23). Dried specimens were rotary-shadowed with platinum at an angle of 6°, and stabilized with carbon. The carbon-platinum replica was floated off the mica sheet and picked up from below on a 300 mesh copper grid. Electron micrographs were taken on a JEOL 100 CX microscope at × 17,000, × 23,000, or × 28,000.

The extent of antibody binding to myosin in platinum-shadowed specimens is detailed in Table I. Fields of well spread myosin molecules were photographed at × 23,000 and negatives were enlarged 2.6-fold. Myosin-II molecules were scored for attached antibody and compared to molecules incubated with control antibodies against chicken skeletal muscle myosin that does not bind to myosin-II (10). Approximately 1.6% of myosin molecules complexed with control antibodies (Table I), but binding was to random regions of the myosin molecule. Such random binding of specific antibodies was observed with the same low frequency. Frequency of specific binding shown in Table I refers to binding to the sites depicted in Figs. 1, 2, and 3. Typically ~25% of the myosin molecules were not scored because they lacked sufficient contrast, lacked some morphological feature characteristic of myosin, or were parts of aggregates in which individual molecules were not discernable.

The distance between the tip of the myosin-II tail and the antibody M2.4 binding site was measured on prints enlarged × 6.05 with a lupe that had a scale interval of 0.1 mm.

Negative Staining and Electron Microscopy: Specimens were stained on glow-discharged, Formvar-coated, carbon-coated grids with 1% uranyl acetate (18, 19). Electron micrographs were taken on a JEOL 100 CX microscope at magnifications of 23,000–46,000. The periodicity of Fab M2.3 decoration of myosin filament bare zones was measured on prints enlarged × 2.60 with the lupe described above.

Filament Formation: To evaluate antibody effects on the polymerization of myosin-II into thin filaments, we incubated 0.5 μM myosin-II with 2 μM antibody in 300 mM KCl, 10 mM imidazole, pH 7.0 (a salt concentration that prevents polymerization) for 30 min and then diluted 20-fold into 20 mM KCl, 10 mM imidazole, pH 6.8, conditions that cause myosin-II to form filaments. After 30 s, 4 μl of the diluted sample was placed on a grid and negatively stained.

The effects of antibodies on the Mg^{++}-induced aggregation of thin myosin-II filaments into thick myosin-II filaments was tested in two ways. Starting with monomeric myosin preincubated with antibody, samples were diluted into 20 mM KCl, 10 mM $MgCl_2$, and 10 mM imidazole, pH 7.0, conditions that favor the formation of the thick myosin-II filaments. Alternatively, myosin-II thin filaments in 20 mM KCl were preincubated with antibody and $MgCl_2$ was added to 10 mM. Samples were negatively stained.

RESULTS

Localization of Antibody-binding Sites on Individual Myosin-II Molecules

Platinum-shadowed preparations of antibody-myosin complexes reveal the location of seven antibody-binding sites on myosin-II. By combining these electron microscopic observations with the data from antibody staining of one-dimensional peptide maps and from competitive binding studies (10), we establish the binding sites of 16 of the 23 monoclonal antibodies.

Antibodies That Bind to the Myosin-II Head

Antibodies M2.1 and M2.10 bind on or close to the myosin-II heads. Platinum-shadowed specimens reveal myosin molecules with extra heads and dimers and oligomers formed by head to head contacts of myosin molecules (Figs. 1 and 2, and Table I). It is possible that these antibodies bind on the myosin-II tails, directly adjacent to the heads. However, no clearly resolvable stretches of tail were ever seen between the

TABLE I

Frequency of Antibody Binding to Individual Myosin Molecules

Antibody	Percent molecules with antibody bound	Binding site*	Number counted
	%		
M2.1	14.1	Heads	542
M2.3	96.6	Tip of tail	118
M2.4	63.0	Tail, adjacent to heads	138
M2.10	34.9	Heads	401
M2.12	76.1	Tip of tail	268
Control antibody	1.6	Random	304

* See Figs. 1–3. Data compiled as described in Materials and Methods.

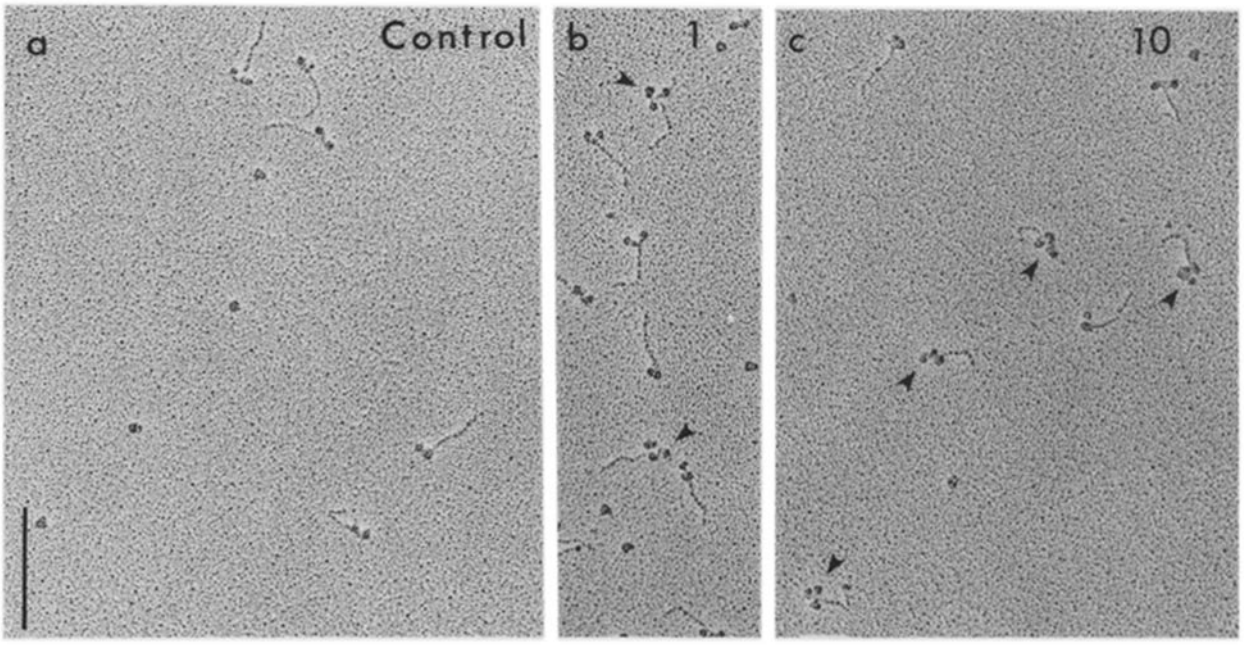

FIGURE 1 Fields of rotary shadowed antibody-myosin-II complexes reveal two antibodies that bind on or near the myosin-II heads. Myosin-II in the presence of (*a*) a control antibody that does not bind to myosin-II, (*b*) antibody M2.1, and (*c*) antibody M2.10. Arrowheads point out some of the antibody–myosin-II complexes and are not meant to distinguish antibody molecules from myosin-II heads. Free antibody and free myosin molecules can be seen in each of the three fields. Bar, 200 nm. × 80,000.

antibodies and the heads. We conclude that both antibodies have binding sites either on the heads or on the tail so close to the heads that it is not feasible to resolve the difference.

Antibodies That Bind to the Proximal End of the Myosin-II Tail Adjacent to Where It Joins the Heads

Antibodies M2.4 (Figs. 2 and 3, and Table I) and M2.7 (data not shown) bind to a region of the myosin-II tail adjacent to where the heads join the tail. Two other antibodies, M2.6 and -26, have not been directly localized by electron microscopy, but we know that they bind to the same region of the myosin-II tail because they stain identical peptides on one-dimensional peptide maps of myosin-II and are sufficiently close to M2.4 and -7 to completely inhibit their binding to myosin-II (10). In the presence of antibody M2.4 many myosin-II molecules appear to have four heads (Fig. 2 and 3). This demonstrates that the stoichiometry of antibody binding can be 2:1. Judging from the length of the tail, the real myosin heads are the two globular structures at the end of the tail. These globular structures bind 71 ± 6 nm (n = 50) from the tip of the tail. Since the tail is ~90 nm long (18), the antibodies must bind ~19 nm from the heads. At lower concentrations of antibody a single antibody molecule binds, so the myosin has three globular structures at the end of the tail. Again, there are no comparable images when myosin-II alone or when myosin-II in the presence of control antibodies are examined (Figs. 1–3).

Antibodies M2.2, -21, -22, and -27 are related but not identical to both M2.4, that binds to the proximal end of the myosin-II tails adjacent to the heads and to M2.10, that binds near or on the heads (10). These antibodies are related because in one-dimensional peptide maps, antibodies M2.2, -4, -10, -21, and -27 all bind to common peptides above ~18,000 mol wt. Similarly, these antibodies bind to the same peptides as M2.22 above ~23,000 mol wt. As a consequence, even though we have not yet localized the binding sites of these antibodies by platinum-shadowing, we can be fairly certain about where they bind on the myosin-II molecule. These 18,000- and 23,000-mol-wt peptides would be 20 and 26 nm long, respectively, if they were alpha-helical coiled coils. Thus these antibodies must bind either to the heads or the proximal 40 nm of the tail.

Antibodies That Bind to the Tip of the Myosin-II Tail

Antibodies M2.3, -9, and -12 bind to the end of the myosin-II tail (Figs. 2 and 3, and Table I). In no case was any myosin tail exposed distal to the antibody. At one end of the tail two myosin-II heads can be distinguished easily in favorable specimens. The other end of the tail usually has a single, globular bulge on it, not seen in preparations of myosin-II alone, myosin-II mixed with nonspecific antibody, or myosin-II in the presence of the 19 other myosin-II specific monoclonal antibodies. Occasionally there are dimers or oligomers of myosin-II that appeared to be myosin molecules linked tail to tail by one or more antibody molecules (Figs. 2 and 3, and Table I). Antibody M2.8 must also bind to the tip of the myosin-II tail because M2.8 specifically inhibits the binding of M2.3 to myosin-II and vice versa, and M2.3 and -8 stain similar constellations of myosin-II peptides (10).

The micrographs suggest that two of these antibodies can bind to each myosin-II tail. The twofold symmetry of myosin suggests that at least two antigenic sites should be available for antibody binding. In favorable specimens the bulge on the tip of the tail appears at least partially resolved into two lobes or domains (Figs. 2 and 3). Further, the mass at the end of the tail appears to vary somewhat, as if sometimes one and at other times two antibodies are bound. Finally, it is sometimes difficult to distinguish between the myosin-II heads and the

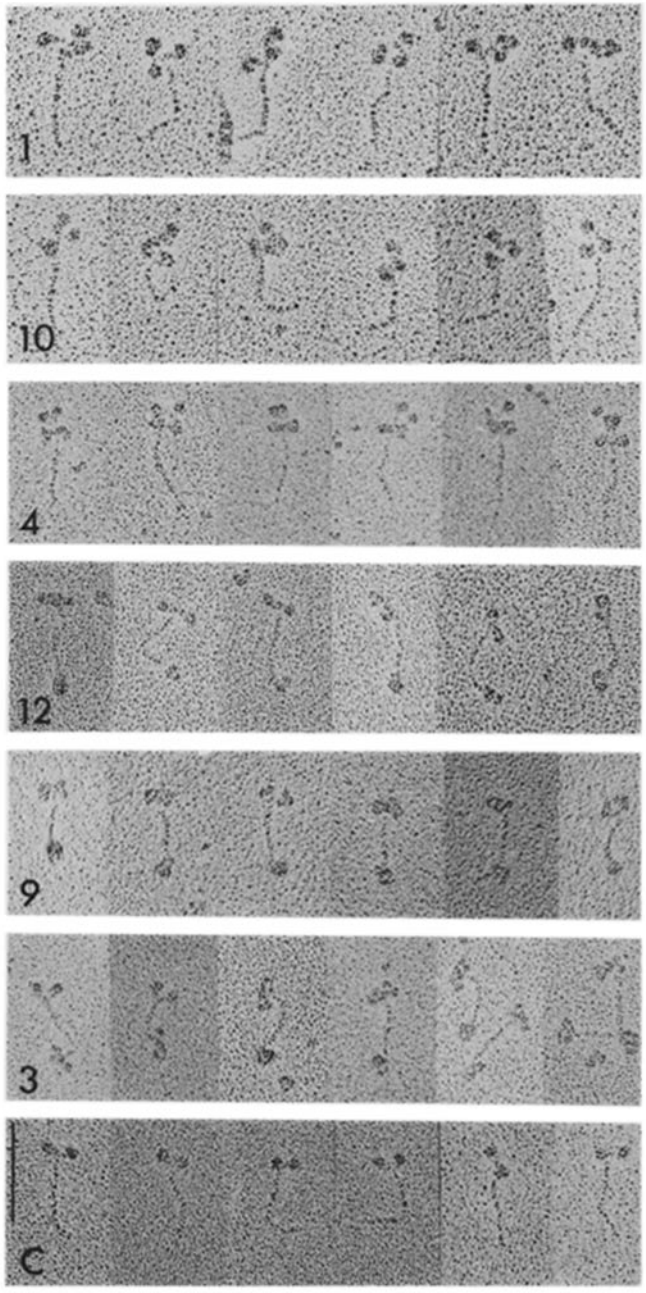

FIGURE 2 Selected electron micrographs of individual antibody–myosin-II complexes. Numbers (M2.X) designate the specific monoclonal antibody used in each preparation. Antibodies M2.1 and -10 bind on or very near the myosin-II heads. Antibody M2.4 binds to the myosin-II tail, ~19 nm from the heads. Antibodies M2.3, -9, and -12 all bind to the tip of the myosin-II tail. The M2.12–myosin-II complexes on the far right demonstrate that it is sometimes difficult to distinguish myosin-II heads from antibodies bound to the tip of the tail. Dimers of myosin-II cross-linked by antibody are shown tail to tail (M2.3-myosin-II complex on the far right). The control (C) is a preparation of myosin-II molecules with a threefold molar excess of Diana, a monoclonal antibody against chicken pectoralis muscle myosin S-1 that does not bind to myosin-II. Bar, 100 nm. × 130,000.

antibodies bound on the opposite end of the myosin-II tail (Fig. 2, especially M2.12, *far right panels*).

Other Antibodies

Antibodies M2.17 and 18 bind to the myosin-II head by biochemical criteria (10) but we have been unable to localize the binding of these antibodies to myosin-II by the platinum- and rotary-shadowing techniques. We have also been unable to localize the binding of the family that includes M2.5, -16, and -19 or the family that includes M2.13, -15, and -20. Antibody M2.11 stains alpha-chymotryptic peptide maps in a pattern identical to the staining pattern seen with M2.1. Unfortunately the smallest peptide stained is a 60,000-mol-wt peptide, so the binding may be in the head or somewhere on the tail.

Antibody Decoration of Myosin-II Filaments

Electron microscopy of myosin-II filaments decorated with monoclonal antibodies provides additional information about the binding sites of some of the antibodies (Figs. 4 and 5).

Control antibodies such as one that binds to skeletal muscle myosin, but not myosin-II, do not affect the appearance of the myosin-II filaments, except the background uniformly accumulates less stain (Fig. 4).

Antibody Binding to the Ends of the Bipolar Myosin-II Filaments

Antibodies M2.1, -4, -10, -17, -18, and -26 bind to the myosin-heads projecting from the back-bone of the bipolar myosin-II filaments on either side of the bare zone (Fig. 4, data not shown for M2.17 and -26). The ends of the filaments are more massive than undecorated filaments, whether or not control antibodies that do not bind to myosin-II are present (Fig. 4*a*). In no case did decoration of the ends of the bipolar filaments accentuate the 15 nm periodicity of the myosin heads (18). Bipolar filaments whose ends were decorated with antibodies were indistinguishable from filaments formed from myosin-II monomers in the presence of the same antibodies. Fig. 4 also shows filaments decorated with M2.1 (Fig. 4*c*) and filaments formed in the presence of M2.1 (Fig. 4*b*). In all other cases where the antibodies decorate the ends of the bipolar filaments such as M2.4, -10, -17, -18, and -26 the filaments can also form from complexes of myosin-II and antibody (Fig. 4).

These observations confirm localization by platinum shadowing and by peptide mapping. They establish that the binding site for M2.1 is found in the amino-terminal region of the 105,000-mol-wt heavy chain peptide (3, 10). In addition they are consistent with biochemical evidence that M2.17 and -18 bind to a 70,000-mol-wt tryptic peptide of myosin-II that includes the amino-terminus of myosin and is part of the myosin head (10). They demonstrate that M2.17 and -18 can bind to myosin heads when the myosin is assembled into filaments. Finally, the similarity between filaments decorated with M2.1, -4, and -10 shows that negatively stained filament-antibody complexes can provide only approximate information about binding sites near the myosin-II heads. The resolution is not sufficient to distinguish between antibodies that bind to the head and those that bind to the tail close to the head.

Antibody Binding to the Bare Zone of the Myosin-II Filaments

Antibodies M2.3, -9, and Fab fragments of M2.3 decorate the bare zone of preformed myosin-II filaments with striking periodicity (Fig. 5). Fab M2.3 molecules bind at an interval of 13.8 ± 1.6 nm (n = 93) with decoration on either side of the filament at each interval, indicating that at least two

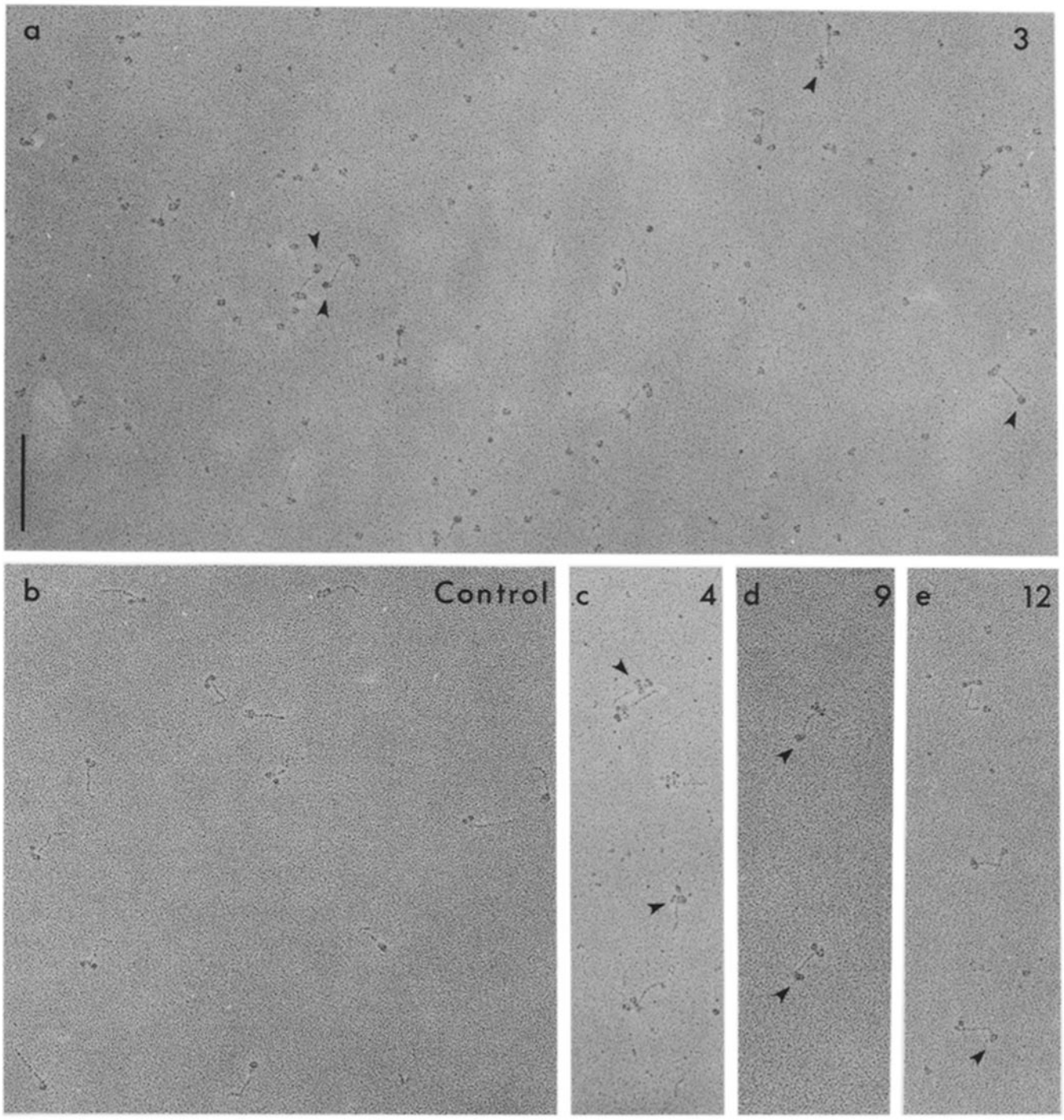

FIGURE 3 Fields of rotary shadowed antibody-myosin-II complexes reveal the location of specific antibody-binding sites on the myosin-II tail. Antibodies M2.3 (*a*), M2.9 (*d*), and M2.12 (*e*) all bind to the tip of the myosin tail. Antibody M2.4 (*c*) binds to the myosin-II tail, ~19 nm from the heads. A control preparation (*b*) of myosin-II alone is identical to myosin-II in the presence of control antibodies that do not bind to myosin-II (compare with Fig. 1*a*). Arrowheads point out some of the antibody–myosin-II complexes. Bar, 200 nm. × 80,000.

myosin molecules are in perfect register. Up to six rows of antibody molecules can be observed in favorable specimens. This is consistent with there being at least 12 myosin-II molecules in each thin bipolar filament (18). In the case of M2.9, decoration results in extensive lateral cross-linking with the antibody molecules forming regular "rungs" in a ladder-like arrangement (data not shown). Antibody M2.12, which also binds to the tip of the myosin-II tail (Figs. 2 and 3), does not appear to decorate the bare zone of the bipolar filaments.

Periodic binding of antibody M2.3 and -9 on the bare zone indicates the longitudinal position of the tips of the myosin-II tails in the myosin-II thin filaments. The 13.8 ± 1.6–nm periodicity is also in close agreement with the 15-nm spacing of myosin-II heads at the ends of the bipolar filaments (18). It shows that the tips of some of the myosin-II tails are exposed on or near the surface of the filament and that at least two myosin molecules are in longitudinal register at each binding site along the filament.

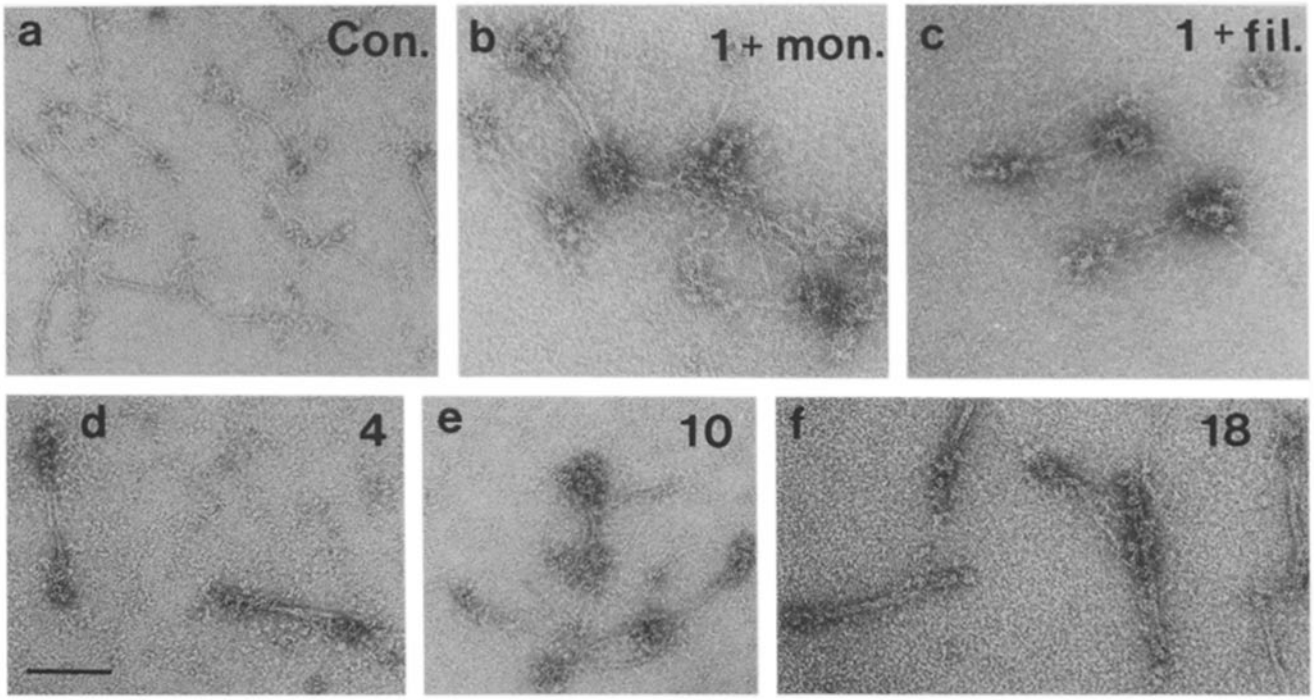

FIGURE 4 Electron micrographs of negatively stained preparations of myosin-II filament-antibody complexes demonstrate that several antibodies bind to the ends of the bipolar myosin filaments. (*b*, *c*, *d*, *e*, and *f*) Decorated filaments formed from monomer in the presence of control antibodies or antibodies M2.1, -4, -10, and -18, respectively. Such decorated filaments were indistinguishable from decorated filaments formed by the addition of antibody to preformed filaments (compare M2.1 decorated filaments in *b* and *c*). Bar, 100 nm. × 109,000.

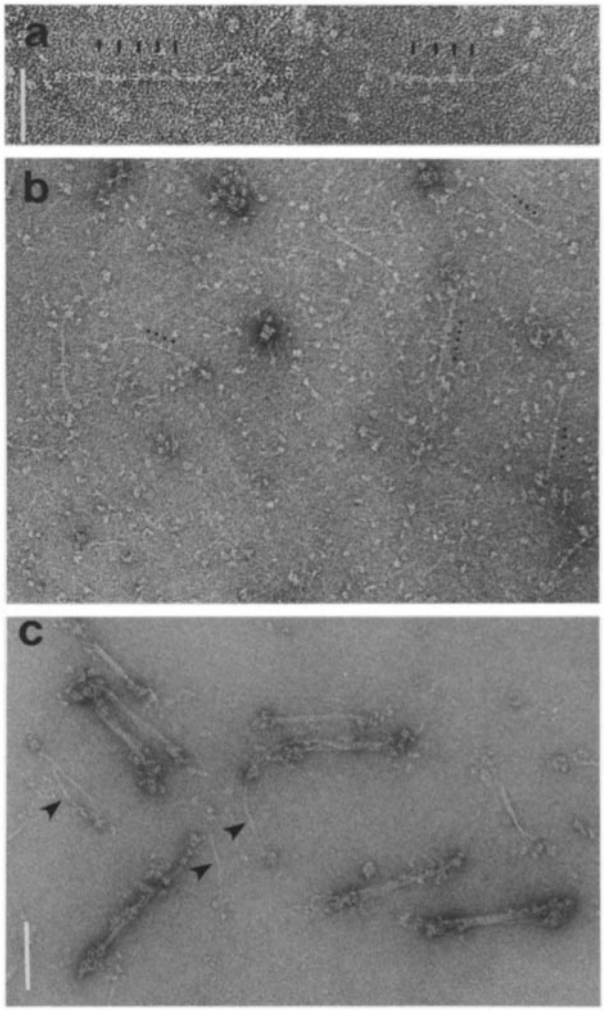

FIGURE 5 Fab fragments of antibody M2.3 decorate the bare zones of bipolar myosin-II thin filaments and prevent the Mg^{++}-induced aggregation of thin myosin-II filaments into thick filaments. (*a*) A high magnification view of negatively stained myosin-II thin filaments decorated with Fab fragments of M2.3. Preformed myosin-II filaments were preincubated with a 4 fold molar excess of Fab fragments of antibody M2.3. Mg^{++}, added to favor the formation of thick myosin-II filaments also slows antibody induced depolymerization of the thin bipolar filaments (12). Hash marks indicate the periodicity of antibody decoration of the thin myosin-II filaments. Bar, 50 nm. × 191,000. (*b*) A low power micrograph of a similar specimen prepared as described in *a*. Fab M2.3 prevents aggregation of thin filaments and decorates their bare zones with 14 nm periodicity (indicated by dots). The wispy appearance of the filaments reflects the ability of M2.3 to induce the slow depolymerization of the myosin-II filaments (12). (*c*) An electron micrograph of a control preparation that contains myosin-II and a fourfold molar excess of Diana, a monoclonal antibody against chicken pectoralis subfragment-1 that does not bind to myosin-II and that does not prevent aggregation of thin filaments into thick filaments upon addition of Mg^{++} to 10 mM. Arrowheads point out undecorated thin bipolar myosin-II filaments (in equilibrium with thick filaments) for comparison to antibody-decorated filament in *a* and *b*. (*b* and *c*) Bar, 100 nm. × 87,000.

Effect of Monoclonal Antibodies on Myosin-II Polymerization

Most of the antibodies have no effect on polymerization. Monomeric myosin-II was preincubated with a fourfold excess of antibody or Fab fragments, the salt concentration was lowered to favor assembly and specimens were negatively stained in preparation for electron microscopy. The absolute concentration of antibody was 2 μM, a concentration at least 50-fold greater than the apparent dissociation constant we measured for these antibodies (10). Normal filaments formed

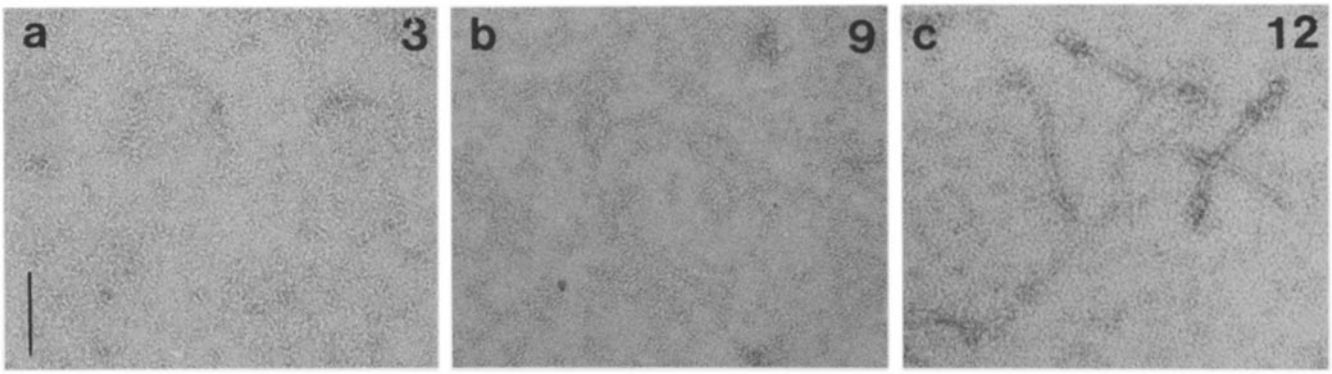

FIGURE 6 Antibodies M2.3 and M2.9 block myosin-II polymerization. Monomeric myosin was preincubated with a 4 fold molar excess of antibody and then diluted into 20 mM KCl to cause polymerization. (a and *b*) Antibodies M2.3 and M2.9, which bind to the tip of the myosin tail, blocked filament formation. Filaments were never seen on any of the grids prepared. (c) In contrast, antibody M2.12, another antibody that binds to the tip of the tail, allows normal polymerization. Filaments also form normally in the presence of control antibodies that do not bind to myosin-II, other antibodies that bind to the tail near the heads (M2.4), and antibodies that bind to the heads (M2.1, 10 and 18, see Fig. 4). In these experiments, normal filaments appeared on all grid squares examined. Bar, 100 nm. × 110,000.

in the presence of antibodies M2.1, -2, -4, -10, -12, -15, -17, -18, -19, -22, -26, and control antibodies that do not bind to myosin-II. Filaments were observed on all grids inspected (see Figs. 4 and 6 for representative micrographs). Thus, antibodies tested that bind to the myosin heads or to the myosin tail near its junction with the heads do not inhibit polymerization. Similar results were obtained in solutions containing 20 mM KCl with or without 10 mM $MgCl_2$. Thin myosin-II filaments formed in the absence of $MgCl_2$ and thick myosin-II filaments formed in 10 mM $MgCl_2$.

In contrast, two antibodies, M2.3 and -9, are potent inhibitors of myosin-II filament formation (Fig. 6). No filaments were ever observed when monomeric myosin-II was preincubated with antibodies M2.3, -9, or Fab fragments of M2.3 before lowering the salt concentration to conditions that would, in the absence of antibody, cause filament formation. This inhibition of assembly occurs in 1, 7, and 10 mM $MgCl_2$. Similarly, M2.3, Fab M2.3, and M2.9 all prevent the side to side aggregation of preformed myosin-II thin filaments into thick myosin-II filaments upon the addition of 7 mM $MgCl_2$ (Fig. 5). None of the other antibodies tested prevented the formation of thick filaments when diluted into 10 mM $MgCl_2$ so we conclude that they do not interfere with the lateral association of thin filaments required to form thick myosin-II filaments. Finally, antibodies M2.3 and -9, and Fab fragments of M2.3 can depolymerize preformed filaments, given sufficient time (12). Antibody-decorated bare zones are only observed when specimens are prepared for electron microscopy at early times following the addition of antibody to preformed filaments.

DISCUSSION

Electron microscopy of myosin-II molecules and filaments reacted with monoclonal antibodies shows directly where the antibodies bind and demonstrates that two of the antibodies that bind to the tail inhibit the polymerization of myosin-II into filaments. These localization studies extend the characterization of the antibodies, reveal information about the arrangement of the myosin-II molecule in the myosin-II filament, and provide a structural framework for the interpretation of the effects of the antibodies on myosin-II function both in vitro and in vivo. By combining the direct, electron microscope localization of seven antibodies with data from peptide mapping and competitive binding experiments, we have established the binding sites of 16 different monoclonal antibodies.

Electron microscopy of antibody-myosin-II complexes directly demonstrate the physical position of the antibody-binding sites on myosin, while staining of peptide maps or the competitive binding experiments can provide more subtle information about these sites. For example, electron microscopy establishes that antibodies M2.3, -9, and -12 all bind at the end of the tail, but cannot distinguish between their binding sites. Antibody staining of one-dimensional peptide maps (10) provides somewhat better resolution; while M2.3, -9, and -12 stain similar constellations of peptides on some of the maps (M2.3 and -9 are similar on hydroxylamine and cyanogen bromide maps, M2.9 and -12 are similar on alpha-chymotryptic maps; see Table III in reference 10), and staining of the other maps by these antibodies results in distinct staining patterns. Competitive binding studies provide the best resolution. None of the three antibodies that bind to the myosin-II tail inhibits the binding of either of the other two. Of the four localization methods, electron microscopy of negatively stained antibody-filament complexes has the lowest resolution, but it provides the only information about the location of the binding sites on myosin-II assembled into filaments. Together the four methods establish the location of the binding sites of all but six of 23 monoclonal antibodies to within 5 or 10 nm as well as much higher resolution information on the relative positions of binding sites within the same region.

Fig. 7 summarizes the localization of the antibody-binding sites on myosin-II. These 16 antibodies are members of four of the six antibody families defined by peptide mapping and competitive binding studies (10). Two families bind to the heads. A large family binds near the junction between the heads and the myosin-II tail. In this region some of the family members bind to the tail, others to the head. The members of the fourth family bind to the tip of the myosin-II tail. We have been unable to localize members of the two other families (M2.5, -16, and -19; M2.13, -15, and -20), or the binding site of M2.11.

Two of four antibodies that bind near the end of the tail inhibit polymerization of myosin-II into thin bipolar filaments, block the Mg^{++}-induced aggregation of thin myosin-II filaments into thick filaments and depolymerize preformed

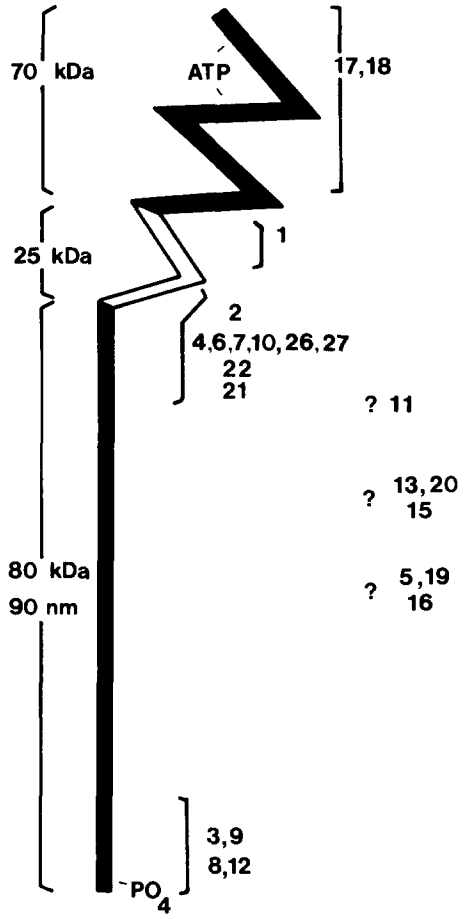

FIGURE 7 A schematic of the myosin-II heavy chain depicts binding sites of the various monoclonal antibodies. PO_4 designates the sites that can be dephosphorylated to allow actin activation of the myosin-II Mg^{++}-ATPase activity. The 105,000-mol-wt carboxy-terminus of the heavy chain is shown divided into an 80,000-mol-wt region that makes up the 90-nm myosin-II tail and a 27,000-mol-wt region that is in the head. ATP indicates that ATP binds somewhere on the 70,000-mol-wt amino terminal fragment. Antibody numbers and brackets show their approximate binding sites.

thin or thick filaments (see also reference 12). These antibodies, M2.3 and -9, demonstrate that, as expected, the tail region of myosin-II plays a crucial role in filament formation. These experiments also illustrate that monoclonal antibodies can be surprisingly subtle probes of structure-function relationships. While antibody M2.12 binds to the end of the myosin-II tail by electron microscopy and is, by peptide-staining criteria, a member of the family that includes M2.3 and -9, it does not compete with M2.3 or -9 for binding to myosin-II. Nor does M2.12 block filament formation like M2.3 and -9. The reasons for these differences will require further investigation, but these observations suggest that inhibition of filament formation requires antibody binding to key sites on the myosin-II tail.

One of two mechanisms is probably responsible for antibody inhibition of filament formation. Antibody binding to specific sites on the myosin molecule may prevent myosin assembly into filaments because of gross steric hindrance or because the antibody may alter allosterically the structure of adjacent parts of the tail that are required for filament formation. The observation that Fab fragments of M2.3 can block polymerization demonstrates that inhibition of filament formation is not the result of nonspecific aggregation or precipitation of myosin monomer.

There is little doubt that the end of the tail is especially important for myosin polymerization, because a 9,000-mol-wt region at the carboxy-terminus tip of the myosin-II tail contains three serine residues whose phosphorylation inhibits the assembly of myosin-II into filaments (3, 4, 13). Antibody binding to myosin-II may mimic phosphorylation of the myosin-II tail and prevent filament formation for subtle structural reasons. This region is also important for the polymerization of muscle myosin where a 5,000-mol-wt region at the end of the muscle myosin tail is responsible for the insolubility of light meromyosin at physiological ionic strength (15).

Antibodies in the family that includes M2.4, -6, -7, and -26 bind to the myosin tail near the heads, a region that may be homologous to the "hinge" region of the muscle myosin tail. There is some evidence (18) that myosin-II has either no or, at the most, a short region comparable to muscle myosin subfragment-2. In muscle myosin, this alpha-helical coiled coil region of the myosin rod is ~50 nm long and is separated from light meromyosin by the hinge region. This hinge region contains a proteolytic sensitive site and a region that is structurally less stable than the remainder of the myosin rod (14, 21). The hinge region and the flexible joint between subfragment-2 and the subfragment-1 heads, together with the presumably passive subfragment-2 region allow the heads to spread up to 60 nm from the myosin filament backbone. The assembled myosin monomers are stabilized into bipolar filaments by lateral interactions between light meromyosin, the distal region of the myosin tail. The subfragment-2 region of the muscle myosin molecule is not considered to participate in filament formation, and accordingly the antibodies that bind to this short region of myosin-II do not inhibit polymerization.

We have been unable to localize a number of the antibody-binding sites directly in platinum shadowed specimens. This is consistent with previous attempts to localize the binding of antibodies to myosin and spectrin. Claviez et al. (2) report localization of five of 24 monoclonal antibodies directed against *Dictyostelium* myosin. Burke and Shotton (1) were able to localize one of four monoclonal antibodies directed against spectrin. We do not known why the binding sites of certain antibodies are not localized by our techniques, but speculate that preparation of the antibody-myosin complexes for platinum shadowing might disrupt antibody binding to the myosin molecule. Winklemann et al. (23) observed that minimizing the time between specimen dilution and shadowing improves preservation of myosin-antibody complexes. We suspect that dilution, the presence of glycerol required for successful specimen preparation and the adsorption of specimens to mica may all contribute to poor preservation of intact antibody-myosin complexes.

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