Monoclonal Antibodies Binding to the Tail of *Dictyostelium discoideum* Myosin: Their Effects on Antiparallel and Parallel Assembly and Actin-activated ATPase Activity

Kathryn Pagh and Guenther Gerisch
Max-Planck-Institut für Biochemie, D-8033 Martinsried, Federal Republic of Germany

**Abstract.** Eight monoclonal antibodies that bind to specific sites on the tail of *Dictyostelium discoideum* myosin were tested for their effects on polymerization and ATPase activity. Two antibodies that bind close to the myosin heads inhibited actin activation of the ATPase either partially or completely, without having an effect on polymerization. Two other antibodies bind to sites within the distal portion of the tail that has been shown, by cleavage mapping, to be important for polymerization. One of these antibodies binds close to the sites of heavy chain phosphorylation which is known to regulate both myosin polymerization and actin-activated ATPase activity. Both antibodies showed strong inhibition of polymerization accompanied by complete inhibition of the actin-activated ATPase activity.

A unique effect was obtained with an antibody that binds to the end of the myosin tail. This antibody prevented the formation of bipolar filaments. It caused myosin to assemble into unipolar filaments with heads at one end and the antibody molecules at the other. Only at concentrations higher than required for its effect on polymerization did this antibody show substantial inhibition of the actin-activated ATPase. These results indicate that, using a monoclonal antibody as a blocking agent, parallel assembly of myosin can be dissected out from antiparallel association, and that essentially normal actin-activated ATPase activity could be obtained after significant reductions in filament size.

**During** cell aggregation of *Dictyostelium discoideum*, amebas respond chemotactically to gradients of cAMP which guide them towards aggregation centers. They respond within seconds to a local stimulus of the attractant by extending pseudopods (Gerisch et al., 1975; Swanson and Taylor, 1982). In response to a sudden increase in cAMP concentration, amebas change their shape with a contraction-like cringe (Futrelle et al., 1982). Amebas also modulate their net speed of movement when exposed to temporal decreases or increases in cAMP concentrations (Varnum et al., 1985).

Myosin has been implicated to function in these cAMP-induced shape changes and movements of *D. discoideum* amebas because it undergoes changes in distribution and in its state of phosphorylation after a chemotactic stimulus. When cells round up with a cringe, myosin that appeared to be filamentous and located throughout the cytoplasm, redistributes to beneath the membrane (Yumura and Fukui, 1985). Myosin seems to be excluded from pseudopods (Rubino et al., 1984) and concentrated at the rear of cells that are migrating up a concentration gradient of chemoattractant (Yumura et al., 1984).

When a pulse of cAMP is given to cells in suspension, threonine residues on the heavy chains of myosin are first slightly dephosphorylated and then rephosphorylated (Rahmsdorf et al., 1978; Malchow et al., 1981; Maruta et al., 1983; Berlot et al., 1985) and the light chains are phosphorylated (Berlot et al., 1985). Heavy chain phosphorylation has two effects on myosin functions in vitro. It reduces the capability of myosin to polymerize (KuczmarSKI and Spudich, 1980) and lowers actin-activated ATPase activity (KuczmarSKI and Spudich, 1980; Maruta et al., 1983).

The relationships between heavy chain phosphorylation, polymerization, and actin-activated ATPase activity of myosin have also been studied in Acanthamoeba. When Acanthamoeba myosin II is phosphorylated at the end of its tail, the actin-activated ATPase activity is inhibited (Collins et al., 1982a, b). Experiments using monoclonal antibodies (Kiehart and Pollard, 1984a) and mild proteolytic digestion (Kuznicki et al., 1985) indicated that tail-bearing myosin II must be filamentous to show actin-activated enzyme activity. However, phosphorylation does not simply inhibit actin-activated ATPase activity by preventing filament formation; phosphorylated myosin II still can polymerize, although the filaments formed are smaller than those of dephosphorylated myosin (Pollard, 1982; Collins et al., 1982b).

Using a threonine-specific myosin heavy chain kinase from aggregation competent *D. discoideum* cells (Maruta et al., 1983) and monoclonal antibodies whose binding sites on the myosin tail had been mapped by electron microscopy...
Materials and Methods

Protein Purification

*Dictyostelium* myosin was purified from aggregation-competent cells as was described by Maruta et al. (1983), using an extraction buffer containing 30 mM Tris-Cl, pH 7.5, 30% sucrose, 0.2 mM ATP, 2 mM EGTA, 1 mM dithiothreitol, 0.02% NaN₃, 10 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride, or one in which ATP was at 0.5 mM and EGTA was replaced by 2 mM EDTA. Purified myosin was stored on ice and used within 3 wk after purification for the ATPase assays. The phosphate content of myosin from aggregation-competent cells was shown by Maruta et al. (1983) to be negligible. Contamination of purified myosin with RNA, which has been shown to affect its polymerization (Stewart and Spudich, 1979), was measured spectrophotometrically and found to be undetectable. In addition, RNase treatment according to Stewart and Spudich (1979) had no effect on myosin polymerization as is described here.

F-actin was prepared from rabbit skeletal muscle (Schleicher et al., 1984) and was generously provided to us by Dr. G. Isenberg.

Monoclonal antibodies, designated as mAb 14-26-5, 21-32-3, 21-51-3, 15-153-33, and 21-96-3, were produced against a chymotryptic tail fragment, while mAb 40-253-6 and mAb 56-396-5 were obtained by screening hybridomas from mice immunized with plasma membrane fractions. Those of mAb 396, mAb 153, and mAb 253, and mAb 55 are shown in Fig. 1, a-f. The antibody binding sites are located within diverse regions of the myosin heavy chain (Fig. 1 g). None of these antibodies competed with one another in solid-phase radioimmunossay. One antibody, mAb 96, binds near to the sites phosphorylated by a myosin kinase (Claviez et al., 1982), we showed that heavy chain phosphorylation sites are located ~150 nm along the length of the 188-nm tail away from the heads and adjacent to a region of the molecule that is important for polymerization of chymotryptic fragments (Pagh et al., 1984). This result suggested that, in *Dictyostelium*, heavy chain phosphorylation directly affects the assembly state of myosin. Spudich and co-workers identified three monoclonal antibodies each of which binds to the tail of *Dictyostelium* myosin and inhibits one of three myosin functions assayed: polymerization, actin-activation of ATPase activity, or motility of myosin-coated beads (Peltz et al., 1985; Flicker et al., 1985). Here we compare the effects on myosin polymerization and actin-activated ATPase activity of eight monoclonal antibodies that bind at defined sites along the tail. A unique effect was found with an antibody that binds to the end of the tail. This antibody completely blocked antiparallel myosin assembly as it normally occurs in the middle region of a filament. The small unipolar filaments that formed could exhibit substantial actin-activated ATPase activity.

ATPase and Sedimentation Assays

Monomeric myosin was preincubated with monoclonal antibody for 2–4 h on ice in high-salt buffer containing 500 mM KCl, 10 mM Tris-Cl, pH 7.5, 0.02% NaN₃, and 1 mM dithiothreitol. For preincubation of preformed myosin filaments with antibody, monomeric myosin was first induced to polymerize by dilution into low-salt buffer containing 10 mM Tris-Cl, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, and 0.02% NaN₃ for 10 min on ice. Subsequently, antibody was added for 4–6 h of preincubation on ice. Protein concentrations were determined according to Lowry for myosin and, by absorption at 280 nm for antibody assuming an extinction coefficient of 1.5. Typically, myosin was at 1 mg/ml and antibody was at 1.5–2 mg/ml during preincubations.

After preincubation of either monomeric myosin or filaments with antibody, the mixtures were diluted 6–10-fold to final concentrations of 40–50 μg myosin/ml with polymerization buffer containing 10 mM Tris-Cl, pH 7.5, 0.2 mM ATP and, unless specified otherwise, 10 mM MgCl₂, diluted from a 4.8 M stock solution as supplied by Sigma Chemical Co. (St. Louis, MO). KC₁ was adjusted to a final concentration of 50 mM. ATPase assays and sedimentation analyses were carried out 1 h after ice.
Figure I. Binding sites of monoclonal antibodies on the tail of D. discoideum myosin. Complexes of monomeric myosin and antibody were rotary-shadowed (a–f). The map of binding sites shown in g has been compiled from previously published (Claviez et al., 1982; Pagh et al., 1984) as well as new data. A new antibody, mAb 396, bound simultaneously to two sites at the same position of the myosin tail (a and b), indicating that identical epitopes on the two heavy chains of the myosin molecule were accessible. These sites were located 14.4 ± 5.4% of the length of the tail away from the heads. The binding sites of three other antibodies important for this paper are shown in c–f. MAb 96 and mAb 55 were distinguishable after double labeling with Fab fragments and IgG, respectively (d). MAb 55 bound to the very end of the tail (e), and binding sites on both myosin heavy chains were simultaneously accessible (f).

Figure 2. Sedimentation of myosin preincubated with antibodies. Monomers (a and b) were preincubated with or without (−) antibodies in high-salt buffer. Filaments (c) were preincubated with the antibody in low-salt buffer. The samples were then diluted into polymerization buffer. After incubation for 1 h (a and b) or 6 and 48 h (c), they were ultracentrifuged for 15 min at 30 psi in an airfuge. Supernatant (S) and pellet (P) fractions were subjected to SDS PAGE in minislab gels and the proteins stained with Coomassie Blue. Control samples in c for total myosin and antibody (T) were not ultracentrifuged. The upper major band is that of the heavy chains of myosin and the lower ones correspond to the heavy chains of the IgG antibody. MAb 396 was poorly soluble under the conditions used; it was the only antibody that pelleted by forming a precipitate, in addition to sedimenting with myosin.

heavy chain kinase (Pagh et al., 1984). Its binding site relative to mAb 55 was demonstrated in a double labeling experiment in which mAb 96 was used as a Fab fragment (Fig. 1 d).

The effects of antibodies on myosin polymerization were assessed by sedimentation experiments. Monomeric myosin in a buffer containing 500 mM KCl was preincubated with a fivefold molar excess of antibody, and then induced to polymerize by dilution into a low-salt, high Mg²⁺ buffer ("polymerization buffer") containing final concentrations of 50 mM KCl, 10 mM MgCl₂, and 0.2 mM ATP. After ultracentrifugation in an airfuge at 30 psi for 15 min, nearly all of the control myosin was found in the pelleted fraction (Fig. 2). Myosin preincubated with antibody sedimented as in the control
Figure 3. Negative stain preparations of myosin preincubated with a fivefold molar excess of antibodies and induced to polymerize in their presence (b–g). In the control (a), no antibody was added. After 1 h in the polymerization buffer, samples were fixed with glutaraldehyde and stained with 1% uranyl acetate. The inset in c illustrates the enhanced 14.5-nm axial periodicity of preformed filaments after incubation with mAb 396.
in all cases (Fig. 2 a) except for three antibodies. About 50 and 96% of the myosin remained in the supernatant with mAb 253 and mAb 96, respectively (Fig. 2 b). About 10% of the myosin preincubated with mAb 55 remained in the supernatant fraction. When ultracentrifugation was carried out at 25 psi for 15 min, up to 40% of the myosin preincubated with mAb 55 was found in the supernatant, indicating that the filaments were smaller than in controls, in which only trace amounts of myosin remained in the supernatant under these conditions.

In contrast to their strong effects on the polymerization of monomeric myosin, neither mAb 55 nor mAb 96 disassembled preformed filaments at a fivefold molar excess over myosin. Even after 48 h of incubation with mAb 96, sedimentation analysis showed very little myosin in the supernatant fraction (Fig. 2 c).

Effects of Antibodies on the Size and Structure of Myosin Filaments

In negative stain preparations, control myosin formed bipolar filaments (Fig. 3 a) that averaged 512 nm in length and formed a bare zone of ~220 nm (Table I), as has been described by Clarke and Spudich (1974). Filaments with sizes resembling the control were found when myosin polymerized in the presence of antibodies that bound nearest to the heads: mAb 32 (Fig. 3 b; Table I) and mAb 51. Because mAb 32 co-sedimented significantly with myosin filaments (Fig. 2 a), its failure to interfere with polymerization could not be due to a weak affinity for myosin.

With mAb 396, a third antibody that binds relatively near to the heads, the filamentous material that formed had prominent lateral striations with a repeat spacing of 14.5-15.0 nm (Fig. 3 c). This is the normal staggering of myosin molecules in filaments (Harrington and Rodgers, 1984). When preformed filaments were incubated with mAb 396, they remained intact and the striations were more clearly seen (Fig. 3 c, inset).

Filaments, which were smaller than in the control, formed in the presence of the two antibodies, mAb 26 and mAb 153, that bind one-third of the length of the tail from the heads (Fig. 3 d). Filament lengths with mAb 26 averaged 360 nm, which is comparable in size to the filaments formed in controls when MgCl2 was only 0.2 mM, rather than 10 mM (Table I). The "apparent bare zone", i.e., the zone that was free of heads and antibodies, was less than the 188-nm tail length because the antibodies remained bound to the filamentous myosin.

The three antibodies with binding sites within the terminal third of the tail had striking inhibitory effects on myosin polymerization (Fig. 3, e-g). Only few filaments were formed in the presence of mAb 253 (Fig. 3 e). This antibody also disassembled preformed filaments, as judged from a reduction in the number of filaments in negative stain preparations after 4 h of incubation with a fivefold molar excess of the antibody. With mAb 96, essentially no filaments were observed in negative stain preparations (Fig. 3 f). As revealed by rotary shadowing, ~85% of the monomeric myosin molecules had bound mAb 96. The remaining monomers may not have formed filaments because they were below a critical concentration required for polymerization. Essentially no dimers or oligomers of myosin were seen, indicating that mAb 96 inhibits the primary steps required for myosin assembly. With mAb 55, an antibody that binds to the tip of the myosin tail, numerous myosin filaments were visible (Fig. 3 g). However, these filaments measured only ~230 nm in length, with an apparent bare zone of 117 nm (Table I), suggesting that they were unusual in structure.

Myosin Forms Small Unipolar Filaments in the Presence of mAb 55 That Binds to the Tip of the Tail

In rotary-shadowed preparations of control samples, the heads of myosin molecules were recognizable at both ends of a filament (Fig. 4, a and b). In contrast to these bipolar filaments, the small filaments formed after incubation with mAb 55 had heads clearly visible at only one end (Fig. 4, c-e). At the opposite end were globular particles somewhat larger than myosin heads, which were comparable in size to unbound antibody molecules seen in the background. In some cases, they appeared to be staggered with the periodic 14-15-nm repeat of myosin filaments (Fig. 4 c), indicating that the structures observed were not produced by cross-linkage of myosin molecules with the antibody. The number of pairs of heads in these filaments varied between 6 and 12.

Evidence that the globular structures seen at one end of the small filaments were antibody molecules was provided by immunogold labeling. The labeling patterns obtained with various dilutions of gold-conjugated anti-mouse antibody are given in Table II and illustrated in Fig. 5, a-g. Most of the labeling was unipolar, i.e., gold particles were located at only one end of the filament. At the highest concentrations of the gold-conjugated antibody used, 90% of all filaments were labeled, and in 90% of these, labeling was unipolar. The remaining 10% of filaments were labeled at both ends although the number of gold particles was always minor at one of these ends (Fig. 5 d). Few filaments were associated with gold particles when preformed filaments were incubated with anti-mouse antiserum alone. Association of gold particles with myosin filaments, when it was observed in these controls, occurred with equal probability either somewhere along the bare zone, or at either of the two filament ends (Table II), indicating the absence of any localized labeling without first antibody.

The unipolar organization of filaments formed in the presence of mAb 55 was striking when the filaments had aggregated by their heads into clusters of three or more (Fig. 5, f and g). Filaments formed in the presence of mAb 32, an antibody that did not alter polymerization, gave a bipolar

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**Table I. Sizes of Myosin Filaments Formed at a Fivefold Molar Excess of Antibodies**

| mAb     | Mg2+ (mM) | Length (nm) | Apparent bare zone (nm) |
|---------|-----------|-------------|-------------------------|
| None    | 10        | 512 ± 58    | 221 ± 24                |
| 32      | 10        | 441 ± 69    | 202 ± 25                |
| 26      | 10        | 360 ± 34    | 154 ± 19                |
| 55      | 10        | 233 ± 20    | 117 ± 17                |
| None    | 0.2       | 360 ± 30    | 208 ± 21                |

Myosin was preincubated with one of the antibodies or with buffer alone. Polymerization was induced by dilution into polymerization buffer with MgCl2 concentrations as indicated. The reaction was stopped after 1 h by the addition of glutaraldehyde. Means and standard deviations are given.
labeling pattern (Fig. 5, h–j; Table II), and they aggregated into chains rather than into clusters (Fig. 5 h). Even the smaller filaments formed in the presence of mAb 26 were found to be bipolar in labeling (Table II).

Effects of the Antibodies on Actin-activated MgATPase Activity Compared with Their Effects on Myosin Polymerization

Sensitivity of the actin-activated ATPase activity to antibodies was studied under the same conditions as used for polymerization of myosin except that F-actin was added to the polymerization buffer. The actin did not change the inhibitory effects of mAb 96 and mAb 55 on polymerization of the myosin. (For other antibodies this was not tested.) Enzyme activity was near maximal at pH 7.5 and 25°C in our standard polymerization buffer, which contained 10 mM MgCl₂ (Fig. 6).

In a first series of experiments, antibody was incubated in high-salt buffer at a fivefold molar excess with monomeric myosin and, after 1 h in the polymerization buffer, samples were assayed for actin-activated MgATPase activities. As shown in Table III (experiments I and II), all antibodies inhibited the enzyme activity to some extent. The degree of inhibition sometimes varied with the amount of F-actin added to the assay mixture. Three antibodies were distinguished from the others by their strong inhibitory effects. Two of them, mAb 253 and mAb 96, bound within the distal portion of the tail of the myosin and were strong blockers of its polymerization (Figs. 2 b and 3, e and f). The third antibody, mAb 396, bound near to the heads and did not block polymerization (Figs. 2 a and 3 c).

When incubated with myosin filaments, only some of the antibodies showed similarly strong inhibition of the actin-activated ATPase activity as when monomeric myosin was applied (Table III, experiment 3). The two best blockers among these antibodies, mAb 32 and 396, did not inhibit the polymerization of myosin (Figs. 2 a and 3, b and c), although mAb 396 was found to be incorporated into preformed filaments (Fig. 3 c, insert). The lack of an effect of mAb 96 on the actin-activated ATPase activity of filaments was in agreement with the inability of this antibody to bind to polymerized myosin (Fig. 2 c). The finding that even after 24 h of incubation the ATPase was only weakly inhibited (Table III,

### Table II. Localization of Monoclonal Antibodies Associated with Myosin Filaments by the Immunogold Labeling Method

| First antibody (mAb) | Immunogold* (rel. concentration) | Labeling pattern† | Filaments labeled§ |
|----------------------|----------------------------------|-------------------|-------------------|
|                      |                                  | Unipolar | Bipolar | Other |                  |
| 55                   | 1                                | 92      | 2       | 6     | <50               |
| 55                   | 2                                | 87      | 11      | 2     | 80                |
| 55                   | 3.3                              | 85      | 13      | 2     | >90               |
| 55                   | 5                                | 90      | 8       | 2     | >90               |
| 32                   | 2                                | 28      | 70      | 2     | 72                |
| 26                   | 2                                | 31      | 62      | 7     | <50               |
| None                 | 2                                | 47      | 4       | 49    | <20               |

Monomeric myosin was incubated with a fivefold molar excess of monoclonal antibody and then induced to polymerize as described in Materials and Methods. For the experiments with mAb 55, filamentous myosin was centrifuged in an airfuge for 30 min at 30 psi. The resuspended filaments were fixed for 10 min with 0.025% glutaraldehyde, ultracentrifuged, and washed with 50 mM ammonium formate, pH 7.2. The fixation greatly reduced the reactivity with mAb 32 and mAb 26; therefore, for these antibodies the fixation step was omitted. We have confirmed that the unipolar labeling pattern obtained with mAb 55 also occurred with unfixed material.

* 20-nm gold particles coated with goat anti–mouse antibody were centrifuged, washed with 50 mM ammonium formate, pH 7.2, and resuspended in the original volume. Numbers denote relative concentrations with 1 representing 10% of the commercial preparation.
† "Unipolar" means that gold particles were at one end of a filament and "bipolar" corresponds to labeling at both ends. "Other" designates labeling within the bare zone of filaments. In each experiment, between 50 and 75 labeled filaments were characterized.
§ Percentage of filaments that were labeled with gold particles.
Figure 6. Myosin ATPase activity as a function of MgCl2 concentrations. Basal (○) and actin-activated (●) ATPase activities were measured in polymerization buffer with varying concentrations of MgCl2.

have preincubated five antibodies at different molar ratios with monomeric myosin. 1 h after dilution into polymerization buffer, myosin was assayed for basal and actin-activated MgATPase activity, and, in parallel, samples were processed for SDS PAGE (Fig. 7).

Each of the five antibodies showed significant effects on either actin-activated ATPase activity or polymerization of myosin, or on both, at an equimolar concentration relative to myosin. Thus, differences between the effects of these antibodies on myosin were not simply caused by varying affinities. None of the antibodies had a significant effect on the basal MgATPase activity with the possible exception of mAb 96, which seemed to partially inhibit this enzyme activity. Over the entire concentration range examined, no effect of mAb 32 and mAb 396 on myosin polymerization was observed (Fig. 7, a and b). These antibodies differed in their inhibitory effect on the actin-activated ATPase, which reached a plateau at a level of ~70% inhibition with mAb

![Figure 5. Localization by immunogold labeling of monoclonal antibodies on filaments. Monomeric myosin was induced to polymerize with a fivefold molar excess of either mAb 55 (a–g) or mAb 32 (h–j). After 1 h, specimens were fixed with glutaraldehyde, ultracentrifuged to remove unbound antibody, and reacted with gold anti-mouse antibody.](image)

**Table III. Actin-activated ATPase Activity of Myosin after Preincubation of Monomers (Exps. 1 and 2) or Filaments (Exps. 3 and 4) with Antibodies**

| mAb | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 4 |
|-----|--------|--------|--------|--------|
|     | ATPase activity | % Inhibition | ATPase activity | % Inhibition | ATPase activity | % Inhibition | ATPase activity | % Inhibition |
| 32  | 102 ± 25 | 49     | 56 ± 3  | 55     | 30 ± 1  | 77     | 39 ± 2  | 73     |
| 31  | 117 ± 16 | 42     | 82 ± 3  | 34     | 103 ± 5 | 20     | ND     | –      |
| 396 | 36 ± 2   | 82     | 14 ± 1  | 89     | 48 ± 3  | 63     | 50 ± 2  | 65     |
| 26  | 203 ± 9  | 0      | 72 ± 1  | 42     | 128 ± 4 | 0      | 147 ± 5 | 0      |
| 153 | 179 ± 22 | 11     | 56 ± 5  | 55     | 129 ± 10| 0      | ND     | –      |
| 253 | 54 ± 5   | 73     | 23 ± 1  | 82     | 101 ± 3 | 21     | 87 ± 1  | 39     |
| 96  | 32 ± 6   | 84     | 14 ± 1  | 89     | 137 ± 5 | 0      | 128 ± 4 | 11     |
| 55  | 124 ± 9  | 38     | 40 ± 2  | 68     | 136 ± 4 | 0      | 154 ± 5 | 0      |
| Control | 200 ± 18 | –     | 125 ± 8 | –      | 128 ± 5 | –      | 143 ± 3 | –      |

After preincubation of myosin with a fivefold molar excess of antibody in high-salt buffer the samples were diluted sixfold into polymerization buffer. ATPase activities are expressed in nmoles ATP hydrolyzed per minute and milligram myosin. Means and standard deviations are given. In Exp. 1, 20 μg of myosin was supplemented with 45 μg of F-actin, which resulted in a 20-fold activation over the basal Mg2+ ATPase activity of the control. In Exp. 2, 15 μg of F-actin was added, resulting in a 10-fold activation over basal enzyme levels of the control. In Exps. 3 and 4, 20 μg of filamentous myosin was preincubated with a fivefold excess of antibodies, and 15 μg of F-actin was added before assay. In Exp. 3, samples were kept for 1 h and in Exp. 4, for 24 h in polymerization buffer before assay. ND, not determined.
Table IV. Synopsis of the Antibody–Myosin Interactions

| No. of antibody | Binding site (% of tail length) | Estimates of $K_D$ | Inhibition of polymerization | Binding to polymerized filaments | Inhibition of actin-activated ATPase† | Starting with unpolymerized myosin | With preformed filaments | Specific properties of the antibody |
|----------------|--------------------------------|--------------------|-----------------------------|----------------------------------|------------------------------------|-------------------------------|---------------------------|----------------------------------|
| 32             | 3                              | $>4 \times 10^{-6}$ | −                           | +                                | +                                  | ++                           | ++                        | Enhances periodic structure of filaments |
| 51             | 9                              | $\geq 3 \times 10^{-7}$ | −                           | ND                               | −                                  | −                            | −                         | Reduce size of bipolar filaments |
| 396            | 14                             | ND                  | −                           | +                                | ++                                 | +++                         | +++                       | Block myosin polymerization completely |
| 26             | 27                             | ND                  | Partial                     | +                                | − to +                             | −                            | −                         | Blocks antiparallel assembly |
| 153            | 31                             | $1.6 \times 10^{-6}$ | Partial                     | +                                | − to +                             | −                            | −                         | |
| 253            | 68                             | ND                  | Complete                    | +                                | ++                                 | +                            | −                         | |
| 96             | 81                             | $\sim 1 \times 10^{-7}$ | Complete                   | −                                | ++                                 | −                            | −                         | |
| 55             | 100                            | $7 \times 10^{-7}$  | Partial                     | −                                | +                                  | −                            | −                         | |

ND, not determined.

* $K_D$ estimates were obtained by counting antibody–myosin complexes and free myosin after rotary shadowing in the electron microscope (Claviez et al., 1982). $K_D$ was calculated for the interaction of a single binding site on the antibody IgG with a single epitope on the myosin tail. Upper and lower limits refer to cases in which one antibody molecule inhibited the binding of a second, identical one to a myosin molecule.

† Indicates inhibition of actin-activated ATPase activity. Variations observed with different concentrations of actin (Table III, Exps. 1 and 2) are included.

Discussion

Selective Inhibition of Antiparallel Myosin Assembly

Monoclonal antibodies with unique binding sites on the tail of *D. discoideum* were found to interfere with filament formation in three distinct ways; by (a) suppressing selectively the antiparallel association of myosin monomers, (b) inhibiting myosin–myosin polymerization completely, and (c) limiting the size but not the normal bipolar structure of filaments.

Two general statements can be made about the effects of antibodies on the actin-activated MgATPase activity of *D. discoideum* myosin. First, any antibody that blocked myosin assembly abolished actin activation, although not every antibody that inhibited actin activation of the ATPase had a concomitant effect on the assembly of myosin. Second, the actin-activated ATPase activity was only marginally affected by reduction in the size of myosin filaments from a length of 500 nm to $\sim 230$ nm, even when antiparallel assembly was eliminated. The effects of the eight antibodies examined are summarized in Table IV.
Figure 7. Basal (○) and actin-activated (●) ATPase activities and sedimentation after preincubation of myosin with antibodies at different ratios. Monomeric myosin was preincubated in high-salt

buffer in the presence or absence of one of the five antibodies as indicated, and the mixtures were diluted 10-fold into polymerization buffer. After 1 h, ATPase activity was measured with or without the addition of F-actin. In parallel samples, without actin, the myosin heavy chain content was determined in total fractions, and in supernatants and pellets obtained by ultracentrifugation for 15 min at 30 psi in an airfuge. The fractions were subjected to SDS PAGE, Coomassie Blue staining, and densitometry, and the pelleted myosin expressed in percent of the total. In a control, showing that monomeric myosin was not precipitated by mAb 55, a sample was ultracentrifuged at the end of preincubation in high-salt buffer (△ in e).

Complete Inhibition of Myosin Assembly by Antibodies Binding to a Region Important for Polymerization

We defined a region important for polymerization of D. discoideum myosin by mapping polymerizable chymotryptic tail fragments with monoclonal antibodies (Pagh et al., 1984). This region spans maximally from ~50 to 80% of the tail length from the heads. The distal portion of this region is adjacent to, and probably covers, the two threonine residues which are phosphorylatable by myosin heavy chain kinase (Maruta et al., 1983).

In the present study, two antibodies were found to completely inhibit myosin–myosin interactions as judged from sedimentation analyses and electron microscopy. These antibodies bind within the region of the tail that we identified as being important for polymerization. One of the two antibodies, mAb 253, binds to a position that is ~68% of the tail length from the heads. The second antibody, mAb 96, binds to a site 81% of the length of the tail from the heads (Claviez...
et al., 1982) which is near to the heavy chain phosphorylation sites (Pagh et al., 1984). It has been shown that only exceptionally more than one molecule of mAb 96 binds to a myosin molecule and that this antibody does not cross-link myosin molecules (Claviez et al., 1982). Our results indicate that the 1:1 complexes of myosin and mAb 96 cannot polymerize at all. With decreasing ratios of antibody to myosin, more myosin molecules remain free of antibody and accordingly form normal filaments. Peltz et al. (1985) also identified an antibody that blocks polymerization of D. discoideum myosin; its binding site appears to lie in between the two binding sites described here (Flicker et al., 1985).

Based on thermodynamic studies of filament formation, only ~1% of the skeletal muscle myosin molecule is assumed to participate in strong associations (Harrington, 1979). Proteolytic removal of as little as 5–10 kD of the tail tip of skeletal muscle myosin (Lu et al., 1983) or Acanthamoeba myosin II (Kuznicki et al., 1985) abolishes polymerization. The finding that antibodies that bind very close to one another on the tail of Acanthamoeba myosin II may or may not inhibit filament formation has suggested that the ability of myosin to polymerize may reside in one or two specific sites (Kiehart et al., 1984). Our results together with those of Peltz et al. (1985) suggest that, in D. discoideum, sites participating in myosin–myosin association may be more dispersed since three separate binding sites of blocking antibodies were identified within a 13% span of the tail. However, monoclonal antibodies are probably not reliable probes for fine mapping of functional sites on proteins. In addition to blocking specific sites, antibodies may cause conformational changes, impose steric constraints, or induce even grosser shape changes, as was suggested from electron microscopic preparations in which tails were seen at positions where an antibody was bound to the myosin tail (Claviez et al., 1982).

Disassembly of myosin filaments is expected to be rapid when the equilibrium between filaments and monomers is shifted (Josephs and Harrington, 1968). Therefore an antibody like mAb 96, which prevents the reassociation of monomers, should efficiently shift this equilibrium. Under our conditions, dissociation seemed to occur very slowly. Even after 48-h incubation with mAb 96 at concentrations sufficient to block reassembly of monomers, only a small fraction of the polymerized myosin was disassembled. In comparison with conditions thought to be physiological ones, we have used a rather high Mg2+ concentration of 10 mM because this was required for an optimal stimulation of actin-activated ATPase, and a higher pH to prevent aggregation of antibodies. It will be of interest to know whether the dissociation rate is faster in vivo such that antibodies that efficiently block polymerization can also disassemble preformed filaments.

Size Constraints on Filaments Imposed by Antibodies Binding Near to the Heads

Two antibodies limited the size but did not alter the structure of myosin filaments. These antibodies, mAb 26 and mAb 153, have binding sites near to one another, at approximately one-third of the length of the tail from the heads. Since an adjacent, more proximal portion of the tail is very sensitive to chymotrypsin (Pagh et al., 1984), these binding sites may correspond to the amino-terminal portion of the light meromyosin fragment formed by proteolytic cleavage of myosins. In skeletal muscle myosin, this portion of the tail is packed into the filament shaft (Harrington, 1979). Therefore, it is likely that these antibodies impose steric constraints on the packing.

The three antibodies that bind to the tail closest to the heads, mAbs 32, 51, and 396, had either undetectable effects or had only marginal ones on filament formation. Both mAb 32 and mAb 396 were demonstrated to bind to filaments, which shows that their binding sites are accessible when myosin is polymerized. (For mAb 51 this has not been tested.) Accessibility of binding sites is particularly evident for mAb 396, which enhances the periodic substructure of preformed filaments. A similar effect has been observed with a monoclonal antibody that binds to skeletal muscle myosin (Shimizu et al., 1985). The portion of the tail near to the heads, to which our three antibodies bind, seems to be flexibly linked to the filament shaft, presumably to allow for movements of the heads (Trinick and Elliott, 1979). It is therefore reasonable that binding of antibody to this portion of the tail does not impose steric constraints on the packing of myosin molecules into filaments.

Effects of Antibodies on Actin-activated ATPase Activity

Antibodies that bind within the proximal 15% of the tail inhibited actin-activated MgATPase activity without preventing myosin from forming large, bipolar filaments. The effect of mAb 32, which binds closest to the heads, saturated without reaching full inhibition of the ATPase, while mAb 396 inhibited completely. The sensitivity of this enzyme activity to monoclonal antibodies binding to the proximal part of the tail has been noted previously (Kiehart and Pollard, 1984b; Peltz et al., 1985), and attention has been drawn to a putative hinge in this region which may function in mechanochemical force transduction (Harrington and Rodgers, 1984).

Two of the antibodies studied, mAb 26 and mAb 153, bind more distal from the heads. Decreases in the length of bipolar myosin filaments from 500 nm to ~360 nm, as caused by these antibodies, had minor effects on the actin-activated ATPase activity.

Strong inhibition of actin-activated MgATPase activity by mAb 96 and mAb 253 paralleled their inhibition of myosin polymerization (Fig. 7). Since these antibodies bind at distances from the heads of 128 and 152 nm, respectively, a direct effect on the ATPase catalytic or F-actin binding sites of the same monomer is unlikely. This finding is in agreement with the work of Kiehart and Pollard (1984b) who recognized, for Acanthamoeba myosin II, a relationship between the inhibitory effects of monoclonal antibodies on filament formation and actin-activated MgATPase. Accordingly, these authors proposed that myosin must be filamentous to show actin-activated ATPase activity as also was suggested by Kuznicki et al. (1985). However, it has to be taken into account that the ATPase activity of nonpolymerizable fragments of D. discoideum myosin is activated by F-actin (Peltz et al., 1981): when myosin is intact, the tail seems to act as a negative regulator, imposing constraints on the activity of the heads.

In D. discoideum, phosphorylation has been reported to reduce filament formation and to inhibit the actin-activated ATPase activity of myosin (Kuczmarski and Spudich, 1980). It is therefore of significance that mAb 96, which binds close to the phosphorylation sites on the myosin tail, inhibits poly-
merization, since it suggests that phosphorylation may also inhibit actin-activated ATPase activity by reducing the capability of myosin to form filaments (Pagh et al., 1984). However, we have shown here, using other antibodies, that the filament size must be reduced drastically before substantial inhibition of actin-activated ATPase activity occurs. It has not yet been shown that heavy chain phosphorylation has that strong effect on polymerization. In *Acanthamoeba*, the filament sizes as well as actin-activated ATPase activities are lower in phosphorylated versus dephosphorylated myosin II (Collins et al., 1982b). But, by varying a number of conditions, Kuzniicki et al. (1983) did not find a strict correlation between filament size and enzyme activity. Thus it is not yet clear, both for *D. discoideum* myosin and *Acanthamoeba* myosin II, to what extent changes in polymerization contribute to the regulation of ATPase activity by heavy chain phosphorylation.

In this context the effects of mAb 55, which binds to the tail at the longest possible distance from the heads, are of interest since its inhibition of bipolar filament formation did not parallel its effect on actin-activated ATPase activity. A sharp decrease in size of filaments from ~500 nm to ~230 nm was accompanied by only slight reductions of actin activation (Fig. 7 e). The gradual decrease in actin-activated MgATPase activity with increasing excess of mAb 55 was apparently not due to further reductions in filament size. It is possible that the increase in number of antibody molecules bound to a maximum of two per myosin molecule affects the interactions, Kuznicki et al. (1983) did not find a strict correlation between filament size and enzyme activity. Thus it is not yet clear, both for *D. discoideum* myosin and *Acanthamoeba* myosin II, to what extent changes in polymerization contribute to the regulation of ATPase activity by heavy chain phosphorylation.

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