Inhibition of Acanthamoeba Actomyosin-II ATPase Activity and Mechanochemical Function by Specific Monoclonal Antibodies

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ABSTRACT Monoclonal and polyclonal antibodies that bind to myosin-II were tested for their ability to inhibit myosin ATPase activity, actomyosin ATPase activity, and contraction of cytoplasmic extracts. Numerous antibodies specifically inhibit the actin activated Mg**-ATPase activity of myosin-II in a dose-dependent fashion, but none blocked the ATPase activity of myosin alone. Control antibodies that do not bind to myosin-II and several specific antibodies that do bind have no effect on the actomyosin-II ATPase activity. In most cases, the saturation of a single antigenic site on the myosin-II heavy chain is sufficient for maximal inhibition of function. Numerous monoclonal antibodies also block the contraction of gelled extracts of Acanthamoeba cytoplasm. No polyclonal antibodies tested inhibited ATPase activity or gel contraction. As expected, most antibodies that block actin-activated ATPase activity also block gel contraction. Exceptions were three antibodies M2.2, -15, and -17, that appear to uncouple the ATPase activity from gel contraction: they block gel contraction without influencing ATPase activity. The mechanisms of inhibition of myosin function depends on the location of the antibody-binding sites. Those inhibitory antibodies that bind to the myosin-II heads presumably block actin binding or essential conformational changes in the myosin heads. A subset of the antibodies that bind to the proximal end of the myosin-II tail inhibit actomyosin-II ATPase activity and gel contraction. Although this part of the molecule is presumably some distance from the ATP and actin-binding sites, these antibody effects suggest that structural domains in this region are directly involved with or coupled to catalysis and energy transduction. A subset of the antibodies that bind to the tip of the myosin-II tail appear to inhibit ATPase activity and contraction through their inhibition of filament formation. They provide strong evidence for a substantial enhancement of the ATPase activity of myosin molecules in filamentous form and suggest that the myosin filaments may be required for cell motility.

There is good evidence that the actin-activated Mg**-ATPase of muscle myosin produces the force for muscle contraction (reviewed in references 5, 37). By analogy, cytoplasmic myosins are likely to generate movement in a variety of nonmuscle systems (16, 36). Mabuchi and Okuno (19) and Kiehart et al. (15) provided the best physiological evidence for the production of force by myosin in a nonmuscle cell. They examined the role of myosin in cytokinesis, nuclear migrations, and chromosome movements by microinjecting antibodies against cytoplasmic myosin into living cells. In vitro, these antibodies inhibited the actin-activated ATPase activity of starfish egg myosin. In the injected cells, antibodies specifically inhibited cytokinesis in an antibody dose-dependent fashion, but the other processes remained unaffected. These experiments showed that cytokinesis requires myosin for force production, and provided evidence against a role for myosin in chromosome movement and nuclear migration.

We have produced and characterized a library of monoclonal antibodies to the two major myosin isoforms from Acanthamoeba to use as probes of myosin function (12). We demonstrated the specificity of antibody binding and determined their relative affinities by evaluating an apparent dis-
sociation constant. Competitive binding studies and antibody staining of one-dimensional peptide maps of myosin-II showed that the antibodies recognize at least 15 unique antigenic determinants that can be grouped into six families whose members bind to sites that are concentrated in six regions of the myosin-II molecule. In a second paper (13), we directly localized the binding sites on myosin-II of some of the antibodies by electron microscopy of platinum-shadowed and of negatively stained specimens. By combining the electron microscope data with the peptide mapping and competitive binding studies, we were able to localize the binding sites of all but seven of the antibodies. Analysis of antibody effects on filament formation demonstrated that two of the 23 antibodies completely inhibited the assembly of myosin-II into filaments, a function likely to be important for the role of myosin in cell motility.

Here we evaluate the effect of monoclonal antibodies on the ATPase activities of myosin-II in vitro and use the monoclonal antibodies to probe myosin function in an in vitro model for cell motility, the contraction of gelled extracts of Acanthamoeba cytoplasm. A subset of the antibodies inhibit the actomyosin-II ATPase activity and contraction of gelled extracts of Acanthamoeba cytoplasm. Three antibodies appear to uncouple gel contraction from ATPase activity; they inhibit contraction without affecting actomyosin ATPase activity. We document the specificity of inhibition of both functions and show that saturation of certain single antigenic sites on the myosin-II heavy chain is sufficient to inhibit mechanochemical function.

The specific mechanism of antibody inhibition of myosin-II function depends on the locus of antibody binding to the myosin-II molecule. Antibodies that bind to the myosin-II heads and block actomyosin-II ATPase activity and gel contraction probably do so by interfering with actin binding or with conformational changes in the head required for actin-activated ATP hydrolysis or cross-bridge cycling. Surprisingly, at least two families of antibodies that inhibit actomyosin-II ATPase activity and gel contraction bind to the myosin-II tail. The first group includes some, but not all, of the antibodies that bind near the junction of the tail with the heads. This is striking evidence that structural domains on the myosin tail, adjacent to where it joins the heads, contribute to force production during cross-bridge cycling. The second group of inhibitory antibodies is a subset of the antibodies that bind to the tip of the myosin-II tail. A number of strong correlations suggest that these antibodies exert their effects through inhibition of myosin-II filament formation (13, 14).

Preliminary accounts of this work were presented at meetings of the American Society for Cell Biology and the Biophysical Society (9–11).

MATERIALS AND METHODS

Materials: Reagent grade chemicals were obtained from the sources described previously (12). Sources for other materials are given in the text.

Proteins: Myosin-II, purified as described previously (12), was dephosphorylated with potato acid phosphatase (Sigma Chemical Co., St. Louis, MO) for 5 h at 29°C in 25% sucrose, 150 mM KCl, 10 mM imidazole-Cl, pH 7.0, 1 mM dithiothreitol, and 1 mM MgCl₂ in the presence of proteolytic inhibitors (10 μg/ml pepstatin, 0.5 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, all obtained from Sigma Chemical Co.) by the method of Collins and Korn (2, 3). To separate dephosphorylated myosin-II from the phosphatase, the preparation was chromatographed on A 15 200–400 mesh (Bio-Rad Laboratories, Richmond, CA) equilibrated with 0.6 M KCl, 10 mM imidazole, pH 7.0, and 5% sucrose. The dephosphorylated myosin-II had <0.8 mol of phosphate per mole of myosin-II by direct chemical assay (29). It was stored in 20% sucrose, 5 mM imidazole-Cl, pH 7.0, 3 mM NaN₃, 1 mM dithiothreitol at 4°C. Sucrose preserves the activity of myosin-II during storage for several weeks.

Monoclonal antibodies were produced, purified, and characterized as described previously (12, 13). 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 (12). Antibodies were routinely stored at 4°C in 20% sucrose, 5 mM imidazole-Cl, pH 7.0, 3 mM NaN₃.

Antibody Fab fragments were made by digesting monoclonal antibodies with papain (32). Monoclonal antibodies (5–13 mg/ml) were mixed with papain (10 μg/mg of antibody ( Worthington Biochemicals, Freehold, NJ) in 10 mM cysteine and 2 mM EDTA, and incubated 12–16 h at 37°C. The preparation was chromatographed on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.1 M sodium phosphate, pH 7.0, 3 mM NaN₃, to separate the Fab and Fc fragments from the papain. The protein peak that contained both antibody fragments was concentrated by dialysis against solid sucrose. The preparation was adjusted to pH 8.0 by addition of sodium phosphate to a final concentration of 0.1 M and chromatographed on Protein A-Sepharose (Pharmacia Fine Chemicals) to remove any contaminant which did not bind to the Protein A column was pooled and concentrated by dialysis against solid sucrose. Fab fragments prepared in this fashion ran as a single ~25,000-mol-wt species on PAGE in SDS.

Protein concentrations were measured by absorbance using extinction coefficients of 0.56 cm⁻¹/ml at 280 nm for myosin-II, 0.62 cm⁻¹/ml at 290 nm for Acanthamoeba actin, and 1.4 cm⁻¹/ml at 280 nm for antibodies and Fab fragments. Methods for SDS PAGE on 4%–15% PAGE (New England Biolabs) and cross-linked polyacrylamide gels appear elsewhere (29).

Myosin-II Concentration in Cytoplasmic Extracts of Acanthamoeba: The myosin-II concentration in Acanthamoeba extracts was measured with a solid-phase antibody-binding assay (12) on nitrocellulose paper. The extract was mixed with SDS sample buffer, boiled, then serially diluted. Duplicate 10 μl samples of each dilution and myosin-II standards in SDS sample buffer were dried onto 1.5-cm squares of nitrocellulose paper. The squares were rinsed several times in 0.1 M Tris-HCl, pH 7.7, 3 mM NaN₃, 0.1% Triton X-100, and 0.1% bovine serum albumin, then incubated with specific antibody (M2.13) against myosin for 2 h at room temperature. After washing with Tris-HCl three times, the amount of specific antibody bound was measured by reaction with ³⁵S-labeled goat anti-mouse antibodies. The squares were washed three times with TSTAB and bound ³⁵S was quantified in a gamma counter. The concentration of myosin-II in the extract was determined by extrapolation from a dilution series of purified myosin treated in parallel with cell extract samples.

Myosin ATPase Activity: The K⁺-EDTA- and Ca⁺⁺-activities of myosin-II were measured at 29°C (30). Inorganic phosphate was determined colorimetrically or by liquid scintillation using trace gamma-labeled ³²P-ATP in an excess of cold carrier ATP. Purified antibodies had no ATPase activity of their own.

Actomyosin-II ATPase Activity: Dephosphorylated myosin-II was used for analysis of antibody effects on actomyosin-II ATPase activity. Under the conditions described below, actomyosin-II Mg⁺⁺-ATPase activity was activated 100–400-fold by 0.5 mM MgCl₂ for each of the five preparations of dephosphorylated myosin-II used for these experiments. ATP hydrolysis due to actin, myosin-II, and antibody alone, was subtracted from samples containing all three proteins to determine the ATPase activity due specifically to actomyosin-II. Hydrolysis of ATP due to actomyosin activity was linear with time and typically accounted for >93% of the inorganic phosphate measured.

Antibody effects on the actomyosin-II ATPase were measured as follows. Equal volumes (typically 20 μl) of myosin-II (0.043 μM) and antibody in their sucrose storage buffers were mixed in small glass culture tubes and preincubated at 0°C for 0.5–2 h. Antibody concentration was adjusted depending on the experiment. The reaction was started by adding 360 μl of a mixture containing appropriate salts, ATP and actin and warming to 29°C. Final solution conditions were 0.15 M KCl, 0.5 mM MgCl₂, 0.1 mM CaCl₂, 0.5 mM KCl, 5 mM Tris·HCl, pH 7.0, 0.1 mM dithiothreitol and 8 to 24 μM actin. The reactions were stopped after 25–35 min by the addition of acid. Inorganic phosphate was determined with gamma labeled ³²P-ATP as described above.

Extracts of Acanthamoeba Cytoplasm: Extracts of Acanthamoeba cytoplasm were prepared with minor modifications of the method described previously (26). Washed cells were homogenized in 1.5 vol of sucrose extraction buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 10 mM ATP, 1 mM EGTA, 1 mM dithiothreitol) with 30 strokes of a Dounce homogenizer with a tight fitting pestle. 10 mM ATP was used instead of 1 mM ATP because the extent of gel contraction was much more reproducible in 10 mM ATP.

Gelation and Gel Contraction: To analyze the effects of antibody on gelation and contraction, we mixed 9 vol of extract with 1 vol of antibody in sucrose buffer (20% sucrose, 5 mM imidazole, pH 7.0, 3 mM NaN₃) in the cold. Antibody concentration was adjusted depending on the experiment.
Samples were preincubated at 4°C for ~30 min, then loaded into 100-μl capillaries that were subsequently sealed at one end with Seal Ease (Clay Adams, Parsippany, NJ). Duplicate capillaries were prepared for each sample and were stored vertically at 4°C. After all the capillaries were prepared, they were transferred to room temperature (22-25°C). Gelation of extract-antibody mixtures was tested using the falling ball assay (22). Following gelation, the samples were stored horizontally at room temperature (22-25°C) and allowed to contract. With time, the clear, gelatin extract became translucent and pulled away from the sealed end of the capillary as it synered a clear fluid. After 10-16 h contraction had ceased and the extent of contraction was measured as the distance the translucent gel pulled away from the seal in the end of the capillary tube. Remarkably, this measure was highly reproducible. In a typical experiment controls containing antibody buffer were contracted 11.3 mm (S.D. = 0.5 mm, n = 6) out of a total gel length of 50-55 mm.

RESULTS

Monoclonal Antibodies Do Not Specifically Inhibit the K⁺-EDTA- and Ca⁺⁺-ATPases of Myosin-II

Only one of the 22 purified monoclonal antibodies to myosin-II inhibits the K⁺-EDTA- or Ca⁺⁺-ATPase activities of myosin-II (data not shown). In these experiments, we preincubated myosin-II with large molar excesses of antibody for 30-60 min at 0°C. In all cases except two, antibody was present in a 10- to 24-fold molar excess over myosin-II. The molar ratios of antibodies M2.9 and M2.13 to myosin-II were four and six, respectively. M2.21 was not tested. Antibody M2.2 inhibited the Ca⁺⁺-ATPase in a linear, concentration-dependent fashion. However, 50% inhibition required a molar ratio of antibody to myosin of approximately 12:1. This suggests that inhibition was not a result of antibody binding to myosin, which should have saturated at a molar ratio of about 1:1 or 2:1. Rather it may have been due to residual contaminants from the ascites fluid. Many of the ascites fluids contained non-antibody materials that inhibited the myosin ATPase or had ATPase activity themselves (data not shown). These contaminants were removed from all of the other antibodies by ammonium sulfate fractionation and DEAE chromatography (12). We conclude that no antibodies specifically inhibited the ATPase activities of myosin-II alone.

Monoclonal Antibodies Inhibit the Actin-activated ATPase of Myosin-II

12 of 19 purified monoclonal antibodies inhibit the actin-activated Mg⁺⁺-ATPase of dephosphorylated myosin-II. We screened the antibodies for their ability to inhibit actomyosin-II ATPase activity using molar ratios of antibody:myosin-II ranging from 23 to 120 (Table I). We preincubated myosin and antibody for at least 30 min at 4°C before adding actin and ATP. Myosin-II samples preincubated with antibody buffer alone resulted in (control) actin activated ATPase rates of ~500 nmol/mg·min when the actin concentration was 0.5 mg/mL. In a molar excess of antibody, where the antibody binding site on the myosin-II would be fully saturated, seven of the monoclonal antibodies did not inhibit the actomyosin-II ATPase activity; neither did Alice, a monoclonal antibody to chicken pectoralis (skeletal) muscle myosin that does not bind to myosin-II. Thus, antibodies inhibit the actin activated ATPase activity only if they bind to specific sites on the myosin-II molecule.

Inhibition of actomyosin-II ATPase activity is not due to proteolysis of myosin-II by components of the various antibody preparations (Fig. 1). We electrophoresed preparations of myosin-II on polyacrylamide gels in SDS to evaluate the polypeptide composition of the myosin-II before and after incubation with the antibodies under conditions used for the actomyosin-II ATPase assays. Incubation with the antibodies did not alter the electrophoretic mobility of either the myosin-II heavy or light chains.

Concentration Dependence of Antibody Inhibition of ATPase Activity

Antibodies inhibit the actin activated ATPase activity of myosin-II in a concentration-dependent fashion (Fig. 2). Plots of actomyosin-II ATPase activity versus antibody concentration show the extent of inhibition and the stoichiometry of antibody to myosin required to cause maximum inhibition for each antibody studied.

Certain antibodies are potent inhibitors (e.g., M2.1, -10, and -27) that block actomyosin-II ATPase activity completely. Many of the antibodies are only partial inhibitors of the actomyosin-II ATPase as manifest by residual, but depressed, ATPase activities even in the presence of a vast excess of

| Table I |
| --- |
| Inhibition of Actomyosin-II ATPase Activity by Molar Excess of Monoclonal Antibody |
| | Molar Ratio | Ratio of Antibody to Actin | Fraction of Control |
| | Antibody | antibody: myosin | concentration (calculated) |
| M2.2 | 1.0 | 0.0 | 0.0 |
| 2 | 0.1 | 0.1 | 0.1 |
| 3 | 0.01 | 0.01 | 0.01 |
| 4 | 0.001 | 0.001 | 0.001 |
| 5 | 0.0001 | 0.0001 | 0.0001 |
| 6 | 0.00001 | 0.00001 | 0.00001 |
| 7 | 0.000001 | 0.000001 | 0.000001 |
| 8 | 0.0000001 | 0.0000001 | 0.0000001 |
| 9 | 0.00000001 | 0.00000001 | 0.00000001 |
| 10 | 0.000000001 | 0.000000001 | 0.000000001 |

* Apparent Kd for each antibody was determined with a solid-phase binding assay. The method and the tabulated values appear in reference 12.
* Free myosin concentration is expressed as a percent of the total myosin in the assay and was calculated assuming that a single antibody molecule binds to a single myosin molecule in simple equilibrium with an apparent dissociation constant as described in reference 12. The actual reaction is likely to be far more complex, but our data (see Figs. 2 and 5) suggests that in all cases where inhibition was observed, binding of a single antibody molecule to myosin is sufficient to completely inhibit actomyosin ATPase activity or gel contraction. Thus we consider our assumption valid to a first approximation. Antibodies M2.8 and -16 are IgMs, and therefore have five times as many binding site equivalents is therefore 37.4 and 96.1, respectively.
* Antibody 21 was not shown in sufficient quantities to allow testing.
FIGURE 1 SDS PAGE of myosin-II alone or myosin-II incubated with antibodies under the same conditions as in the actomyosin ATPase assay. The polypeptide composition of the myosin-II is unaffected by incubation with antibody. (Lanes M) Myosin-II alone. (Numbered lanes) Myosin-II incubated with specific antibody (M2.x). (Lane A) Myosin-II incubated with Alice, a monoclonal antibody against chicken pectoralis myosin subfragment-1 that does not bind to myosin-II. Migration of molecular weight standards are shown to the right of each gel. Numbers at lower right indicate the molecular weights (x10^-3).

inhibitor (e.g., M2.3, -6, and -9). Certain antibodies do not inhibit at all (e.g., Alice and M2.11, see also Table I).

In most cases, maximum inhibition occurs with molar ratios of antibody to myosin-II of close to 1:1. This concentration dependence holds for both partial and complete inhibitors of actomyosin-II ATPase activity (compare M2.1, -10, and -27 with M2.4 and -6) and suggests that inhibition is due to a specific interaction between the purified antibody and myosin. The most significant exceptions are antibodies M2.3 and -19, which require 1.5 and threefold molar excesses of antibody, respectively, for maximal inhibition.

The extent to which each specific antibody inhibitor affects the actomyosin-II ATPase rate varied somewhat. Duplicate preparations assayed at the same time were nearly identical and routinely varied by <2%. However, we analysed the effect of molar excesses of antibodies M2.1 and -3 on the actomyosin-II ATPase activity 10 or more times each, using purified myosin from five different preparations. Inhibition ranged from 84–98% (mean = 87%, S.D. 7%, n = 10) for M2.1 and from 70–90% (mean = 79%, S.D. = 9%, n = 11) for M2.3. Antibody M2.4, a member of the largest family of related antibodies inhibited actomyosin-II ATPase activity 82–98% (mean = 90%, S.D. = 8%, n = 3). Other antibodies including those that sometimes completely inhibited the actomyosin-II ATPase activity displayed similar variability (M2.10, range of inhibition 91–100%, mean = 94%, S.D. = 4%, n = 4). The reason for this variability remains unclear but seems due to the subtle differences in the preparations of Acanthamoeba actin used for these assays.

We investigated the mechanism of antibody inhibition of actomyosin-II ATPase by traditional enzyme kinetic methods (such as reciprocal rate versus reciprocal actin concentration plots as a function of antibody concentrations), but our studies were inconclusive because of the nonlinearity of the plots, even in the absence of antibody (data not shown). This confirms the observations of Collins and Korn (3). Such nonlinearity of the double reciprocal plots is probably because both actin and myosin-II are present as filaments rather than freely diffusing monomers under the conditions used to maximize actin activation of the Mg\(^{2+}\)-ATPase of myosin-II, namely low salt and high Mg\(^{2+}\) (17, 28; and Kiehart, unpublished observations).

Fab Fragments Also Inhibit the Actomyosin-II ATPase

Fab fragments of antibodies M2.1 and -3 inhibit the actomyosin-II ATPase rate. We made Fab fragments of two

FIGURE 2 Inhibition of actomyosin-II ATPase activity is antibody concentration dependent. The antibodies are identified on each graph (M2.x). Myosin-II concentration was 0.43 µM and Acanthamoeba actin concentration was 11.8 µM.

Kiehart and Pollard Acanthamoeba Actomyosin-II 1027
antibodies and tested whether cross-linking of antigenic determinants on the myosin-II heavy chain by bifunctional antibodies was required for inhibition of actomyosin-II ATPase activity. Fab M2.1 was an effective inhibitor of the actomyosin-II ATPase as shown in Fig. 3. Maximal inhibition by antibody M2.1 and by Fab M2.1 occurred at approximately 1:1 ratios of antigen combining sites to myosin peptides. At least twice as many moles of Fab as antibody were required because the whole antibody has two antigen-binding sites, the Fab fragments, only one. Also, the Fab fragments may have a slightly lower affinity for myosin-II and may have been damaged during proteolytic cleavage of the antibody for Fab preparation.

**Antibodies Inhibit Contraction of Gelled Extracts of Acanthamoeba Cytoplasm**

The monoclonal antibodies also inhibit the contraction of gelled cytoplasmic extracts, an in vitro model system for cell motility. When high speed supernatants of sucrose extracts of *Acanthamoeba* cytoplasm are allowed to warm they form a gel. With time the gelled extract becomes translucent and contracts, syneresing a clear fluid phase (Fig. 4). We mixed high speed supernatant of *Acanthamoeba* with each antibody to test their effects on gelation and contraction.

In separate experiments, we used a solid-phase radio-binding assay to estimate that the concentration of myosin-II in the extracts was ~0.24 μM, a value comparable to 0.6 μM in intact cells (data not shown). This value is close to the 0.7-μM value estimated for myosin-II concentration in intact cells by densitometry of SDS gels (31). We describe the effects of the antibodies in terms of their stoichiometry to their target antigen, myosin-II.

None of the monoclonal antibodies inhibit the gelation of *Acanthamoeba* extracts. Within 10 min of warming to room temperature, each sample of extract containing a molar excess of monoclonal antibody formed a gel as verified by the falling ball assay. Controls, which included antibody buffer alone or high concentrations of Alice, an antibody against chicken pectoralis muscle myosin subfragment-1 that does not bind to myosin-II, also had no effect.

14 of 19 monoclonal antibodies tested inhibit gel contraction (Table II). 11 of the 14 inhibitory antibodies also inhibit actomyosin-II ATPase activity. Antibodies M2.2, -15, and -17 did not affect actomyosin-II ATPase activity but inhibit gel contraction. Alice (see above) and several specific mono-
clonal antibodies had no effect on contraction. Some of the antibodies allowed contraction to an intermediate extent.

Antibodies inhibit gel contraction in a concentration-dependent fashion (Fig. 5). We varied the concentration of antibody we added to cell extracts and found that most antibodies inhibited gel contraction maximally when the stoichiometry of specific antibody to extract myosin-II was somewhat less than 1:1. Some antibodies inhibit contraction completely. Other specific antibodies, like control antibodies that do not bind to myosin-II, have no effect. Still other antibodies inhibit contraction only partially, even when there is a large molar excess of antibody over myosin.

Contraction, like actomyosin-II ATPase activity, varied inversely with antibody concentration. We directly compared the antibody concentration dependence of inhibition of actomyosin-II ATPase activity to inhibition of gel contraction in two ways. Fig. 6 shows inhibition of both processes by antibody M2.10. The fraction of control actomyosin-II ATPase activity and control gel contraction are each plotted as a function of the ratio of antibody M2.10 to myosin-II. Remarkably, inhibition of both processes can be described by a single curve. For most of the other antibodies, the concentration dependence of inhibition of actomyosin-II ATPase activity and gel contraction fall on similar but not precisely the same curves (compare Figs. 3 and 5).

Comparison of inhibition of actomyosin-II ATPase activity and of gel contraction for all the antibodies appears in Fig. 7. We plot the molar ratio of antibody to myosin required to inhibit gel contraction to 50% of maximum versus the molar ratio of antibody to myosin required to inhibit actomyosin-II ATPase activity to 50% of maximum. Most antibodies fall into one of two groups. Antibodies that are potent inhibitors of both processes fall near the origin, at the lower left. Those that inhibit neither process appear diagonally opposed, in the upper right. Antibodies M2.2, -15, and -17 are notable exceptions because they block gel contraction without influencing actomyosin-II ATPase activity. Conversely, M2.19 inhibits the actomyosin-II ATPase but does not appear to affect gel contraction.

**DISCUSSION**

Monoclonal Antibodies Are Specific and Potent Inhibitors of Mechanochemical Function

Monoclonal antibodies can be both specific and potent inhibitors of myosin-II function in vitro. They are specific in that antibodies that bind to at least 11 distinct sites on the myosin-II molecule block function, while antibodies that bind to at least four other sites have no influence on myosin-II filament formation (13, 14), actomyosin-II ATPase activity or the contraction of gelled extracts of amoeba cytoplasm. In
ATPase activity. Antibodies which are effective inhibitors of both traction and ATPase activity. A plot of the concentration of antibody vs. inhibition of actomyosin-II ATPase activities for the actomyosin-II ATPase assay appears in Fig. 2. The same antibody required to cause 50% inhibition of actomyosin-II ATPase activity or gel contraction versus the concentration of the antibody (expressed as the ratio of antibody to myosin-II) required to cause 50% inhibition of gel contraction will fall close to the origin, at the lower left. Antibodies which inhibit neither process will fall opposite, at the upper right. Conditions for the actomyosin-II ATPase assay are given in the legend for Fig. 2.

**FIGURE 6** Dependence of actomyosin-II ATPase activity (C) and cytoplasmic extract contraction (O) on the concentration of antibody M2.10. Alice, a monoclonal antibody against chicken pectoralis myosin subfragment-1 that does not bind to myosin-II affects neither actomyosin-II ATPase activity (C), or gel contraction (O). Conditions for the actomyosin-II ATPase assay are given in the legend for Fig. 2.

**FIGURE 7** Graphical comparison of antibody inhibition of gel contraction and ATPase activity. A plot of the concentration of antibody (expressed as the ratio of antibody to myosin-II) required to cause 50% inhibition of gel contraction versus the concentration of the same antibody required to cause 50% inhibition of actomyosin-II ATPase activity. Antibodies which are effective inhibitors of both processes fall close to the origin, at the lower left. Antibodies which inhibit neither process fall opposite, at the upper right. Conditions for the actomyosin-II ATPase assays appear in Fig. 2.

In all cases, the ability of the antibodies to inhibit the function of myosin was tested at antibody concentrations at least 39-fold greater than the apparent dissociation constant for antibody binding to myosin-II (Tables I and II). The apparent $K_D$ was measured with a solid-phase binding assay. The methods and tabulated values for the various antibodies appear in reference 12. Assuming that inhibition requires one antibody molecule bound to myosin (Figs. 2 and 5) we calculate that even in the worst case (highest apparent $K_D$ with lowest concentration of antibody), >97% of the myosin-II was complexed with antibody. On average the concentration of free myosin in these assays was <1% of total myosin-II in the assay. Thus free myosin cannot account for all the actomyosin-II ATPase activity observed in these assays. We conclude that binding of antibody to certain sites on the myosin-II molecule inhibits actomyosin-II ATPase activity or gel contraction, while binding to certain other sites has no observable effect on these functions.

Antibodies to three other sites, M2.2, -15, and -17 have a unique effect: they appear to uncouple contraction from actomyosin-II ATPase activity. We have not localized their binding sites precisely, but M2.2 must bind somewhere near M2.4 because it stains similar constellations of peptides on a number of the peptide maps and M2.17 must bind to the myosin-II heads, because its antigenic determinant is on the 70,000-mol-wt tryptic fragment of the myosin-II heavy chain (13, 14).

The antibodies are potent in that saturation of certain single antigenic sites on each myosin-II heavy chain can completely inhibit polymerization, actomyosin-II ATPase activity and/or gel contraction. These antibodies abolished the ability of myosin to function as a mechanochemical energy transducing protein. In other cases, inhibition was extensive, but not complete: activity decreased with antibody concentration to a certain extent, then plateaued. Again, in most cases, inhibition was nearly maximal when a single antigenic site was saturated with antibody. A relatively constant, residual actomyosin-II ATPase activity or contraction remained, even in the presence of vast molar excesses of antibody. This is classical partial inhibition (34). The antibody hinderers, but does not abolish myosin function, presumably by binding to a site that decreases, but does not completely inhibit, substrate turnover.

Complete inhibition of gel contraction by certain antibodies against myosin-II identifies myosin-II as the major mechanochemical energy-transducing protein in this model of amoeba motility and shows that myosin-I alone cannot contract the gelled extracts. It was already known that a partially purified myosin-II, but not myosin-I, caused contraction of *Acanthamoeba* actin gels reconstituted from purified proteins (23). However, this failure of myosin-I could have been due to the absence of accessory proteins required for myosin-I function. Our experiments with a crude cytoplasmic extract, that is likely to contain all of the accessory proteins, and purified antibodies that bind to myosin-II but not myosin-I, provides even stronger evidence that myosin-II is not sufficient for gel contraction. It is our hope that microinjection of antibodies into living amoebae will answer the more important question of the in vivo functions of myosin-I and -II.

Since none of the antibodies to myosin-II inhibit gelation of the cytoplasmic extracts, it is unlikely that myosin-II is a major actin filament cross-linker in these extracts. Even those antibodies that block myosin-II polymerization (M2.3 and -9; see reference 13) had no effect on time required for gelation. Although it is very likely that myosin-II filaments bridge two or more actin filaments, at least transiently, to generate the tension required for contraction, gelation probably occurs when stable cross-links between actin filaments are formed by the other actin-binding proteins found in the amoeba (22, 23, 27).

**Mechanisms of Myosin-II Inactivation by Antibodies**

The mechanism by which each antibody affects the mechanochemical function of myosin-II depends on exactly
mobility or flexibility of the myosin-II molecule required for direct steric interference with actin binding or with some local effects or long range effects. Local effects might include mechanism of inhibition in more detail.

It will be important to test Fab fragments of two antibodies that bind to opposite ends of the myosin-II molecule (M2.1 and -3) both inhibit actomyosin-II ATPase activity and gel contraction to the same extent as the intact antibodies. It will be important to test Fab fragments of the other antibodies when we study their mechanism of inhibition in more detail.

The other general mechanisms by which the antibodies may influence myosin function fall into two broad categories: local effects or long range effects. Local effects might include direct steric interference with actin binding or with some mobility or flexibility of the myosin-II molecule required for cross-bridge cycling. We tried to evaluate antibody effects on actin binding by myosin-II using a pelleting assay. Unfortunately, part of the complexes of myosin-II and antibody pelleted in the ultracentrifuge even in the absence of actin, so Fab fragments of the antibodies will be required to determine whether or not the antibodies hinder actin binding. We consider inhibition of actin binding a likely mechanism by which at least some of the antibodies block myosin-II function.

Mechanisms of Inhibition by Anti-Head Antibodies

Two antibodies M2.1 and -10, bind close to or on the myosin-II heads as shown by electron microscopy (13), and both are potent inhibitors of actomyosin-II ATPase activity and gel contraction. Electron microscopy, competitive binding studies, and staining of one-dimensional peptide maps all indicate that M2.1 binds to a different part of the heads than M2.10. Because antibody M2.10 competes, albeit poorly, for binding with the core family that includes M2.4, -6, -7, and -26, it is likely that M2.10 binds at the base of the head, close to or even at the junction with the tail (12, 13). Another antibody, M2.27 may bind nearby, because it, like M2.10, inhibits ATPase activity more strongly than other members of the family that includes M2.4, -6, -7, and -26. Antibodies that bind to the head are likely to inhibit the mechanochemical function of myosin-II by either interfering with actin binding or by altering the structure of the head. We cannot distinguish between these mechanisms at this time. It is of course not at all surprising that antibodies directed against the heads exert a profound effect on mechanochemical function by myosin.

In contrast, two other antibodies that bind to the myosin-II heads have no effect on the actin activated ATPase activity of myosin-II. Antibodies M2.17 and -18 bind to the 70,000-mol-wt tryptic fragment of myosin-II and therefore unambiguously bind to the myosin-II heads (12). This peptide can be photoaffinity labeled with ATP in the catalytic site (24), but the specific site where M2.18 binds may not participate in mechanochemical energy transduction. Antibody M2.17 appears to uncouple gel contraction from ATPase activity. It has little influence on ATPase activity, yet inhibits gel contraction (Tables I and II). The lack of inhibition by these two antibodies also suggests that rotational and/or translational diffusion of the myosin-II heads is not rate limiting in the actomyosin-II ATPase reaction. If it were, the greater than twofold increase in the mass of the heads caused by the bound antibodies would surely influence the rate. The mechanism by which M2.17 blocks gel contraction will require further investigation.

Mechanisms of Inhibition by Antibodies That Bind to the Myosin-II Tail Adjacent to the Heads

Three classes of antibodies bind to the myosin-II tail near to where it joins the myosin-II heads. Some antibodies block both actomyosin-II ATPase activity and gel-contraction, one blocks only gel contraction and one inhibits neither actomyosin-II ATPase activity or gel contraction. These three classes of antibodies that bind the tail near the myosin heads must recognize different domains that participate in myosin function in profoundly different ways.

Remarkably, most of the antibodies that bind near the

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**Table III**

Summary of Antibody Binding Sites and the Effect of Antibodies on Myosin-II Function

| Binding site     | Inhibited Function* | Actomyosin-II ATPase activity | Gel contraction |
|------------------|---------------------|------------------------------|-----------------|
| Head             |                     | No effect                    | +               | +               |
| 1, 10            | +                   | +                            | +               |
| 11, 18           |                     | +                            | +               |
| 17               |                     |                              | +               |
| Proximal end of the tail |            |                              | +               | +               |
| 2                | +                   | +                            | +               |
| 4, 6, 7, 26, 27  |                     | +                            | +               |
| 22               |                     |                              | +               |
| Tail             |                     | +                            | +               | +               |
| 3, 9             |                     |                              | +               | +               |
| 12               |                     |                              | +               | +               |
| Unknown          |                     |                              | +               | +               |
| 5, 13, 19, 20    |                     |                              | +               | +               |
| 15               |                     |                              | +               | +               |

* * + * indicates that the function was inhibited.

Filament formation data is from reference 13. Antibody M2.21 has not yet been available in sufficient quantity to test its effect on myosin-II function. Antibodies M2.8 and M2.16 are IgMs and are not soluble under the assay conditions required to test actomyosin-ATPase activity, gel contraction or filament formation. We assayed their effect on actomyosin-II ATPase activity; neither was an inhibitor.
juncture of the heads and the tail of myosin-II (M2.4, -6, -7, -21, -26, and -27) are potent and specific inhibitors of actomyosin-II ATPase activity and gel contraction. By analogy with other myosin molecules, we expect that the actin-binding sites of myosin-II are on a part of the heads distal to their junction with the tail. Consequently, it seems unlikely that antibodies such as M2.4, that bind to the myosin-II tail ~20 nm from the myosin-II heads, interfere directly with actin binding. The simplest interpretation is that these antibodies inhibit ATPase activity and gel contraction through their influence on the structure of myosin in the local region to which they bind. They may lock this region of the molecule into a conformation that prohibits the structural changes necessary for catalytic and contractile function. This implies that in the region to which these antibodies bind, these processes are tightly coupled to conformational changes in the structure of myosin.

Antibody M2.2 is very interesting because like M2.15 and -17 it appears to uncouple contraction from actomyosin-II ATPase activity. We have not localized the binding site precisely, but it must bind somewhere near M2.4, because it stains similar constellations of peptides on a number of the peptide maps. We speculate that the conformational changes on this part of the tail that are required for catalysis differ subtly from those required for contraction. Perhaps the changes required for mechanochemical coupling are much more extensive than those required for catalysis.

Antibody M2.22 binds to another site on the myosin-II tail near the heads but has no effect on ATPase activity or contraction. The localization of this binding site relative to those that influence function should provide important insights into the mechanism of energy transduction by myosin-II.

The tail region of myosin-II adjacent to the heads may be the counterpart of one or both of the presumed hinge regions that link the heads of muscle myosin to subfragment-2 and subfragment-2 to light meromyosin. A special feature of the structure of the myosin-II molecule is that it may have either no or only a very short subfragment-2 region (18, 28). The whole muscle myosin tail is probably an alpha-helical coil (20, 21), but the regions that flank the 50 nm long, stable subfragment-2 segment contain protease-sensitive sites and one displays a quasi-independent thermal instability near physiological temperatures (40). These regions are referred to as hinges because they are flexible enough to allow the myosin heads to rotate fairly freely (38) and the whole subfragment-2/ head region to swing out from the backbone of the thick filament (8, 18, 25). Harrington and his co-workers (7, 40) have even suggested that the subfragment-2/light meromyosin hinge region undergoes conformational changes that result in mechanochemical energy transduction by actomyosin.

These experiments pose some serious questions about whether the myosin heads are fully active energy transducers independent of their normal association with the tail. On one hand, muscle myosin subfragment-1 retains full actomyosin ATPase activity (37) and can generate movements by interacting with actin filaments in in vitro model systems (42; also recently reviewed in references 5, 35). On the other hand, inhibition of both actomyosin-II ATPase activity and gel contraction by antibodies that bind to the proximal end of the myosin-II tail provides strong evidence that this part of the myosin-II tail actively participates in mechanochemical energy transduction. This discrepancy may be explained by fundamental differences in the mechanism of force production by skeletal muscle and cytoplasmic myosins. Models for the mechanism of force production by cytoplasmic myosins (and perhaps by other myosins) have to account for a structurally important site in the proximal end of the myosin-II tail is intimately involved in the ability of myosin to hydrolyze ATP and perform work.

Our observations on the importance of the region of the tail adjacent to the heads are compatible with the helix-coil transition model for force production by myosin (7), that calls on the hinge region to participate in mechanochemical energy transduction. In fact, experiments to test whether the antibodies stabilize the region of the tail adjacent to the heads and prevent the thermal melt and helix-random coil transition could provide direct evidence for this model. The results with these antibodies are also compatible with other models for force production, provided that the antibodies inhibit conformational changes in the tail and thereby allosterically influence the structural changes in the myosin head that occur during cross-bridge cycling.

There is additional evidence that the part of the myosin molecule near the junction of the heads and tail is important in the regulation of myosin. The myosin regulatory light chains have been localized to a site on the myosin head distal to the actin-binding site near where the heads meet the tail (41). Phosphorylation of the myosin regulatory light chains of vertebrate cytoplasmic myosins and smooth muscle myosins influences filament formation, actomyosin ATPase activity and even the gross conformation of the myosin (1, 4, 33, 39). Likewise, Ca++ binding by some invertebrate muscle myosins requires regulatory light chains and regulates the actomyosin ATPase activity (reviewed recently in reference 6).

Mechanism of Inhibition by Anti-Tail Antibodies

There is a good correlation between the ability of an antibody to inhibit filament formation and to inhibit actomyosin-II ATPase activity. Two antibodies, M2.3 and -9, bind to the tip of the myosin tail, inhibit filament formation, actomyosin-II ATPase activity and gel contraction. A third antibody, M2.12, also binds to the tip of the myosin-II tail, but does not inhibit either filament formation or ATPase activity. This evidence suggests that antibodies that bind to the tip of the myosin-II tail probably inhibit ATPase activity and gel contraction through the inhibition of myosin-II filament formation. In experiments described elsewhere, we supply further support for this mechanism by demonstrating a good correlation between the time course of disassembly of preformed myosin-II filaments by antibody M2.3 and the concomitant loss of actomyosin-II ATPase activity (14). The correlation holds under two widely different ionic conditions where filaments differ profoundly in morphology and stability.

The experiments reported here confirm that neither of the two antibodies (M2.3 and -9) that appear to inhibit actomyosin-II ATPase activity by preventing myosin-II filament assembly reduce the ATPase to zero (14). The residual actomyosin-II ATPase activity is probably attributable to depolymerized myosin-II. This means that the depolymerized myosin-II has a specific activity of ~50-100 nmol· min⁻¹· mg⁻¹ in 0.5 mg/ml actin and that assembly into filaments stimulates the activity 5-10-fold. This could be by one of two possible mechanisms. The significance of these findings and
their relevance to other studies on the influence of polymerization of myosin on its actin activated ATPase activity is discussed elsewhere (14).

Myosin-II filaments are probably required for gel contraction. While residual actomyosin-II ATPase activity in the presence of excess antibody M2.3 is ~20% of control values, inhibition of gel contraction by M2.3 is more nearly complete. This suggests that either the actin activated ATPase characteristic of monomeric myosin-II produces forces insufficient for gel contraction or more likely, that bipolar filaments are required for mechanical reasons to generate the force required for gel contraction.

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