

Parallel Modulation of Brush Border Myosin Conformation and Enzyme Activity Induced by Monoclonal Antibodies

Sandra Citi,* Robert A. Cross,* Clive R. Bagshaw,† and John Kendrick-Jones*

*MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom; and †Department of Biochemistry, Leicester University, Leicester LE1 7RH, United Kingdom

Abstract. Monoclonal antibodies binding to distinct epitopes on the tail of brush border myosin were used to modulate the conformation and state of assembly of this myosin. BM1 binds 1:3 of the distance from the tip of the tail to the head and prevents the extended-tail (6S) monomer from folding into the assembly-incompetent folded-tail (10S) state, whereas BM4 binds to the tip of the myosin tail, and induces the myosin to fold into the 10S state. Thus, at physiological ionic strength BM1 promotes and BM4 blocks the assembly of the myosin into filaments. Using BM1 and BM4 together, we were able to prevent both folding and filament assembly, thus locking myosin molecules in the extended-tail 6S monomer conformation at low ionic strength where they normally assemble into filaments.

Using these myosin-antibody complexes, we were able to investigate independently the effects of folding of the myosin tail and assembly into filaments on the myosin MgATPase. The enzymatic activities were measured from the fluorescent profiles during the turnover of the ATP analogue formycin triphosphate (FTP). Extended-tail (6S) myosin molecules had an FTPase activity of $1-5 \times 10^{-3} \text{ s}^{-1}$, either at high ionic strength as a monomer alone or when complexed with antibody, or at low ionic strength as filaments or when maintained as extended-tail monomers by the binding of BM1 and BM4. Folding of the molecules into the 10S state reduced this rate by an order of magnitude, effectively trapping the products of FTP hydrolysis in the active sites.

NONMUSCLE myosins can exist in vitro as monomers with folded tails or with extended tails (sedimentation coefficients 10S and 6S respectively), or as filaments (Scholey et al., 1980; Citi and Kendrick-Jones, 1986). The position of the equilibrium between these conformations is determined by a number of factors including ionic strength, the state of myosin light chain phosphorylation, and the presence of MgATP (Kendrick-Jones et al., 1982; Scholey et al., 1983; Kendrick-Jones et al., 1987). When the myosin adopts the folded conformation, the products of nucleotide hydrolysis are trapped at the active sites (Cross et al., 1986, 1988). In contrast, when the molecules are extended, as for example in $\geq 300 \text{ mM NaCl}$, the rate of release of the products of nucleotide hydrolysis is at least 200-fold faster. Since filamentous myosin released the products of hydrolysis at a rate comparable to that of extended monomer, it was suggested that the folding of the myosin tail was involved in nucleotide trapping (Cross et al., 1988). To test this hypothesis and investigate the role of the myosin tail in the conformational changes that the myosin molecule can undergo (e.g., in folding, unfolding, and filament assembly), we have used two anti-(brush border myosin) monoclonal antibodies. These antibodies bind to epitopes in two regions of the molecule (a) at the tip of the tail (BM4) and (b) $\sim 1:3$ of the distance from the tip of the tail to the head (BM1). In previous studies, these and other antibodies have proved useful in probing the struc-

ture and function of this myosin in vitro (Citi and Kendrick-Jones, 1987; Citi and Kendrick-Jones, 1988) and in vivo (Hoener et al., 1988).

At physiological ionic strength, the antibodies allowed us to block selectively either filament formation or folding or both and to correlate the induced changes in myosin conformation with the changes in the rates of release of the products of nucleotide hydrolysis from the active sites in the myosin heads. The conformation and state of assembly of the myosin in each myosin-antibody complex was established by EM and sedimentation experiments. Measuring the enzymatic activities of these antibody-myosin complexes using formycin triphosphate (Jackson and Bagshaw, 1988a) demonstrated that the trapped state is a property exclusively of folded molecules, and that assembly into filaments per se does not greatly affect the enzymatic activity of the extended myosin molecules.

Materials and Methods

Materials

Formycin triphosphate was prepared as described previously (Jackson and Bagshaw, 1988a). Adenosine triphosphate was obtained from Calbiochem Behring Corp. (San Diego, CA). All other chemicals (enzyme grade) were obtained from British Drug Houses (Poole, Dorset, U.K.).

Preparation of Proteins

Myosin was prepared from chicken intestinal epithelial cells and dephosphorylated using purified chicken gizzard myosin light chain phosphatase, as described previously (Citi and Kendrick-Jones, 1986).

Monoclonal anti-(brush border myosin) antibodies were purified from hybridoma culture supernatants as described in Citi and Kendrick-Jones (1987). The pure antibodies (at concentrations in the range of 25–100 μM), in 150 mM NaCl, 25 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, 1 mM NaN_3 , pH 7.3 (PBS) were stored at 4°C (short term) or at -20°C (long term). The position of the binding site of each antibody on the myosin rod is shown schematically in Fig. 1 A.

Biochemical Methods

Sedimentation Experiments. To determine the effect of the monoclonal antibodies and Fab fragments on the solubility of brush border myosin, a modification of the sedimentation assay previously described (Kendrick-Jones et al., 1987) was used. Aliquots of nonphosphorylated brush border myosin (in 0.6 M NaCl, 1 mM EGTA, 25 mM Tris-HCl, pH 7.5, 0.1 mM DTT) were mixed in tubes (Eppendorf made by Brinkmann Instruments, Inc., Westbury, NY) with either a small volume of PBS (control with no antibody), or purified monoclonal antibodies or Fab fragments or control Ig (all in PBS), to obtain a final myosin concentration of 0.7 μM and a final antibody concentration of 0.8–3 μM . The mixtures were adjusted to 0.6 M NaCl by the addition of 4 M NaCl if necessary. To half of each mixture 5 mM MgATP was added, and this half was dialyzed separately into 175 mM NaCl, 5 mM MgCl_2 , 2 mM ATP, 1 mM EGTA, 20 mM imidazole, pH 7.2, and 0.1 mM DTT (buffer F). The other halves of each mixture were dialyzed into 175 mM NaCl, 5 mM MgCl_2 , 1 mM EGTA, 20 mM imidazole, pH 7.2, 0.1 mM DTT (buffer F without ATP). After 90-min dialysis, an aliquot of each sample was taken ("total" myosin), and the remaining dialysate was centrifuged at 28 psi (100,000 g) for 20 min in the airfuge. The top two-thirds of each supernatant after centrifugation were carefully pipetted out, and used for the determination of the "soluble" myosin. Because of the presence of the antibodies, the concentration of the myosin could not be measured directly. Thus, identical volumes of the samples before centrifugation (total) and of the supernatants after centrifugation were diluted with equal volumes of SDS sample buffer, and 5–10 μl of each sample were run on 5–20% polyacrylamide gradient SDS gels. The amount of myosin present in each sample was determined by densitometry of the myosin heavy chain bands. The concentration of "soluble" myosin (nonfilamentous myosin remaining in the supernatant after centrifugation at 100,000 g) was then expressed as a percent of the total myosin concentration (Fig. 1 B). To determine the amounts of soluble myosin present in the FTPase assays, these sedimentation experiments were repeated under the same conditions as those used for these assays. The myosin (0.5 μM) and either intact antibody (1.5 μM) or Fab derivative (1 μM) were mixed together at 0.3 M NaCl and incubated in ice for 30 min. They were then diluted to a final concentration of 75 mM NaCl, 2 mM MgCl_2 , 1 mM EGTA, 10 mM imidazole pH 7.0, 0.5 mM DTT in the presence or absence of 1 mM MgATP and thoroughly mixed. Aliquots were taken for "total" myosin samples and the remainder centrifuged in the airfuge at 28 psi for 20 min and processed as described above (Fig. 1 C).

Formycin Triphosphatase (FTP)¹ Assays. Assays using formycin triphosphate were carried out as described by Jackson and Bagshaw (1988a). The myosin-antibody complexes were prepared by mixing nonphosphorylated brush border myosin (in 0.6 M NaCl, 1 mM EGTA, 25 mM Tris-HCl pH 7.5, 0.1 mM DTT) with the purified monoclonal antibodies at 0.3 M NaCl to give a myosin concentration of 0.5 μM and a concentration of each antibody of 1–2 μM . The myosin-antibody complexes and myosin control (without antibody) were incubated at 20°C for 10 min and then in ice for 30 min. They were diluted to a final concentration of 75 mM NaCl, 1 mM EGTA, 2 mM MgCl_2 , 10 mM imidazole pH 7.0, 0.5 mM DTT, and assayed. Fluorescence measurements were made using a ratio recording spectrofluorimeter with a 5-mm path-length cell as described by Jackson and Bagshaw (1988b). Formycin fluorescence was excited at λ 313 nm and the emission monitored at λ 350 nm. Data were digitized as 500 \times 12 bit points using a microcomputer (II+; Apple Computer Inc., NY, NY), and were subsequently transferred to a computer for least squares (300 series; Hewlett-Packard Co., Palo Alto, CA) fitting to a single or double exponential function using the Marquadt algorithm.

Protein Concentrations and Gel Electrophoresis. Protein concentrations during the preparation of myosin and antibodies were measured by the method of Lowry et al. (1951), using BSA as a standard. The concentration

1. *Abbreviation used in this paper:* FTP, formycin triphosphate.

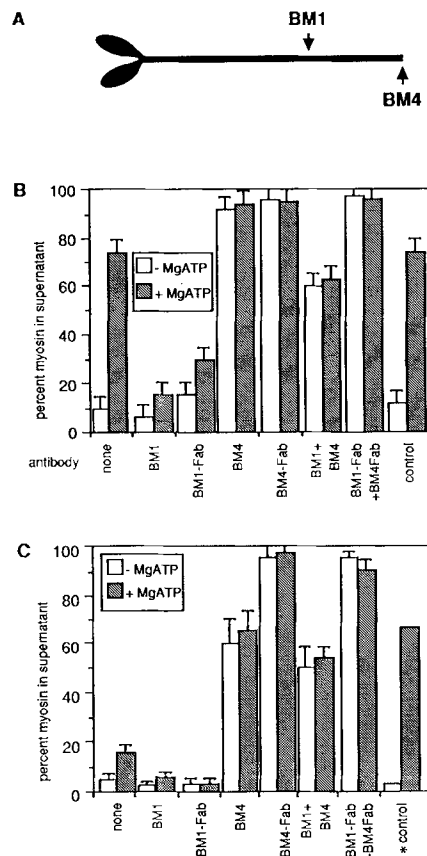


Figure 1. The effects of the monoclonal antibodies on the solubility of brush border myosin. (A) Diagram showing a cartoon of the myosin molecule, with the positions of the binding sites of the two monoclonal antibodies used in this study. (B) Histogram showing the amounts of nonassembled myosin (determined using the sedimentation assay) in the presence of monoclonal antibodies BM1 and BM4 and their respective Fab fragments, in the absence (white bars) and in the presence (shaded bars) of MgATP at 175 mM NaCl. The error bars are standard deviations based on three determinations. Myosin alone and myosin-antibody mixtures (myosin concentration 0.7 μM , antibody concentration 0.8–3 μM) were dialyzed into buffer F (175 mM NaCl, 5 mM MgCl_2 , 1 mM EGTA, 20 mM imidazole, pH 7.2, 0.1 mM DTT, \pm 2 mM MgATP at 4°C). An aliquot of each was taken and the remaining dialysate was centrifuged at 100,000 g, to sediment filaments. The percent of myosin in the supernatant was calculated by comparing the densitometer scans of the myosin heavy chain band (at 200 kD) in the sample before centrifugation (which was taken as 100%) and after centrifugation. *none*, no antibody; *control*, control immunoglobulin. (C) Histogram showing the amounts of nonassembled myosin (determined using the sedimentation assay described above) in the presence of monoclonal antibodies BM1 and BM4 and their respective Fab fragments, in the absence (white bars) and in the presence (shaded bars) of MgATP at 75 mM NaCl; i.e., under conditions as close as possible to those used in the FTPase assays. The myosin (0.5 μM) and either the intact antibodies (1.5 μM) or their Fab derivatives (1 μM) were mixed together at 0.3 M NaCl and incubated on ice for 30 min. They were then diluted to a final concentration of 75 mM NaCl, 2 mM MgCl_2 , 1 mM EGTA, 10 mM imidazole pH 7.0, 0.5 mM DTT in the presence and absence of 1 mM MgATP, thoroughly mixed and processed by the "sedimentation assay" described above. *none*, no antibody and MgATP added to preformed filaments at 75 mM NaCl; **control*, control immunoglobulin and MgATP added to the myosin at high salt before dilution to 75 mM NaCl.

of myosin was also determined spectrophotometrically, using an absorption coefficient ($A_{280\text{ nm}}$) of $0.54\text{ mg}^{-1}\text{ ml}^{-1}\text{ cm}^{-1}$.

PAGE in the presence of SDS was carried out as described by Matsudaira and Burgess (1978), using 5–20% polyacrylamide gradient gels calibrated with myosin heavy chain (relative molecular mass of 200 kD), phosphorylase B (95 kD), albumin (68 kD), skeletal muscle actin (42 kD), and skeletal muscle myosin light chain (25 kD). For densitometry, the gels were stained in a 0.1% wt/vol solution of Coomassie brilliant blue (PAGE Blue 83, BDH) in 50% methanol, 10% acetic acid, destained in 10% methanol, 10% acetic acid, and dried. The gels were scanned using a laser scanning densitometer. Images of the gel were stored and analyzed using an interactive computer program written for the Vax 8600 by Judy Smith (manuscript in preparation).

Electron Microscopy. Folded brush border myosin molecules were prepared by dialyzing a solution of nonphosphorylated myosin ($1\text{ }\mu\text{M}$ in 0.6 M NaCl, 25 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.1 mM DTT) against 175 mM NaCl, 5 mM MgATP, 20 mM imidazole, pH 7.2, 0.1 mM DTT, 0.5 mM EGTA, for 90 min. Previous studies (Kendrick-Jones et al., 1987) have established that the half-time for NaCl equilibration during dialysis is of the order of 10 min. The dialysate was centrifuged for 20 min at 17 psi in an airfuge (Beckman Instruments, Inc., Palo Alto, CA), the supernatant was taken, and the myosin concentration determined by the method of Lowry et al. (1951). To prepare myosin-antibody complexes, aliquots of the supernatant (containing folded myosin molecules) were mixed with a small volume of purified monoclonal antibody ($25\text{--}100\text{ }\mu\text{M}$ in PBS, with 5 mM MgATP added), and each mixture was further diluted, if necessary, with dialysis buffer to obtain a final myosin concentration in the range of $0.4\text{--}0.6\text{ }\mu\text{M}$ and an antibody concentration of $1\text{--}2.5\text{ }\mu\text{M}$. The samples were incubated for 4 h on ice, diluted with an equal volume of glycerol (final myosin concentration $0.2\text{--}0.3\text{ }\mu\text{M}$), mixed thoroughly and sprayed onto freshly cleaved mica for rotary shadowing (Citi and Kendrick-Jones, 1987). The grids were examined in an electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ), operated at 80 kV.

Aliquots of the myosin and myosin-antibody complexes were taken from the FTPase assays and negatively stained as described previously (Citi and Kendrick-Jones, 1986) and examined in the electron microscope.

Results

Perturbation of the Monomer-Polymer Equilibrium of Brush Border Myosin by Monoclonal Antibodies

The effects of the antibodies on the solubility of the myosin in vitro at approximately physiological ionic strength was determined using a sedimentation assay (Fig. 1 B). With $\sim 1\text{ }\mu\text{M}$ nonphosphorylated brush border myosin in the absence of MgATP, $\sim 90\%$ of the myosin was filamentous and thus pelleted, whereas in MgATP only $\sim 25\%$ of the myosin pelleted, and $\sim 75\%$ was in the supernatant (Fig. 1 B). This was in agreement with our previous observations, showing that at physiological ionic strength, MgATP disassembles brush border myosin filaments (Citi and Kendrick-Jones, 1986; Kendrick-Jones et al., 1987). All the antibodies had a specific effect on the assembly of nonphosphorylated brush border myosin filaments, confirming our earlier results using electron microscopy (Citi and Kendrick-Jones, 1988). For example, the binding of BM4 or BM4-Fab to the tip of the myosin tail resulted in a large increase in myosin solubility in the absence of MgATP ($90\text{--}100\%$ of the myosin was soluble instead of $\sim 10\%$), and also a less marked, but significant increase in myosin solubility in the presence of MgATP (from $\sim 75\%$ without the antibody to $\sim 95\%$ in its presence). BM4 does in fact completely inhibit filament assembly (Citi and Kendrick-Jones, 1988). In the absence of MgATP the antibody BM1 and its Fab fragment had little effect on the solubility of the myosin, and the amount of soluble myosin with this antibody was very low, and similar to that obtained with the control (Fig. 1 B). This is in agreement with the observation that BM1 allows filament assembly to occur (Citi and

Kendrick-Jones, 1988). When MgATP was present, BM1 dramatically reduced the amount of soluble myosin, to values similar to those obtained in the absence of MgATP; thus $\sim 90\%$ of the myosin was pelleted (Fig. 1 B). A significant reduction in the amount of soluble myosin was also observed with BM1-Fab (only 20–30% of myosin remained in the supernatant). Although when examined in the electron microscope BM1 plus BM4 or their Fab derivatives completely blocked filament assembly in the presence and absence of MgATP (Table I), in the sedimentation assays $\sim 40\%$ of the myosin pelleted in the presence of both intact antibodies (Fig. 1 B and C). This discrepancy is because of the formation of cross-linked nonfilamentous oligomers that occur in the presence of intact antibodies (see Table I).

Do the Antibodies Inhibit the Folding of the Tail of Brush Border Myosin?

Electron microscopic analysis of the myosin supernatants at approximately physiological ionic strength and in the presence of MgATP showed that $\sim 90\%$ of the soluble myosin ($\sim 70\%$ of the total) was in a folded conformation (Table I). The folding of the tail into three segments generated molecules with a very compact structure in the electron microscope (Fig. 3 A) (see also Citi and Kendrick-Jones, 1986). When BM1 was added to the folded myosin, no folded molecules were seen, and $\sim 90\%$ of the myosin was assembled into a homogeneous population of short ($0.3\text{--}0.4\text{ }\mu\text{m}$) bipolar filaments (Table I, Fig. 2 D). Very occasionally oligomers (one example is shown in Fig. 2 D, c) or unpolymerized extended myosin molecules complexed with the antibodies were seen (Table I). Similar results were obtained when the myosin and antibody were mixed together before dialysis into buffer F (not shown), indicating that the effect of the antibody is independent on whether it is added to the myosin before or after folding. The observation that BM1-Fab had the same effect as BM1 (for example, see Fig. 2 D, d), was in agreement with the sedimentation assay, and was further proof that

Table I. Quantitative Analysis of the Effects of the Antibodies on the Folded Myosin Conformation

	Filamentous	Folded tail	Extended tail	Oligomers
Low salt/ATP				
Control	25	70	2	3
+BM1	92	0	6	2
+BM4	0	59	4	37
+BM1 + BM4	0	1	29	70
High salt				
Control	0	0	98	2

The percent of myosin molecules present as filaments (filamentous) or as monomers with extended tails or folded tails are shown. The values were obtained after examining 200–400 rotary shadowed molecules in electron micrographs at $30,000\times$. The samples for rotary shadowing were prepared as described in Materials and Methods. *High salt* indicates molecules in 600 mM NaCl, 25 mM Tris-HCl, 5 mM Mg^{2+} , 0.1 mM DTT. *Low salt/ATP* indicates buffer F (175 mM NaCl, 5 mM MgCl_2 , 2 mM ATP, 1 mM EGTA, 20 mM imidazole, pH 7.2, 0.1 mM DTT). *Oligomers* includes small aggregates of myosin molecules (present in 600 mM NaCl or in buffer F) or molecules cross-linked by antibodies. In the oligomers with BM4, the myosin molecules were mostly folded (see Fig. 2 B), whereas with BM1 + BM4 they had extended tails (see Fig. 2 C). For BM1, the percent of filamentous myosin was in agreement with the values obtained with the sedimentation assay (Fig. 1).

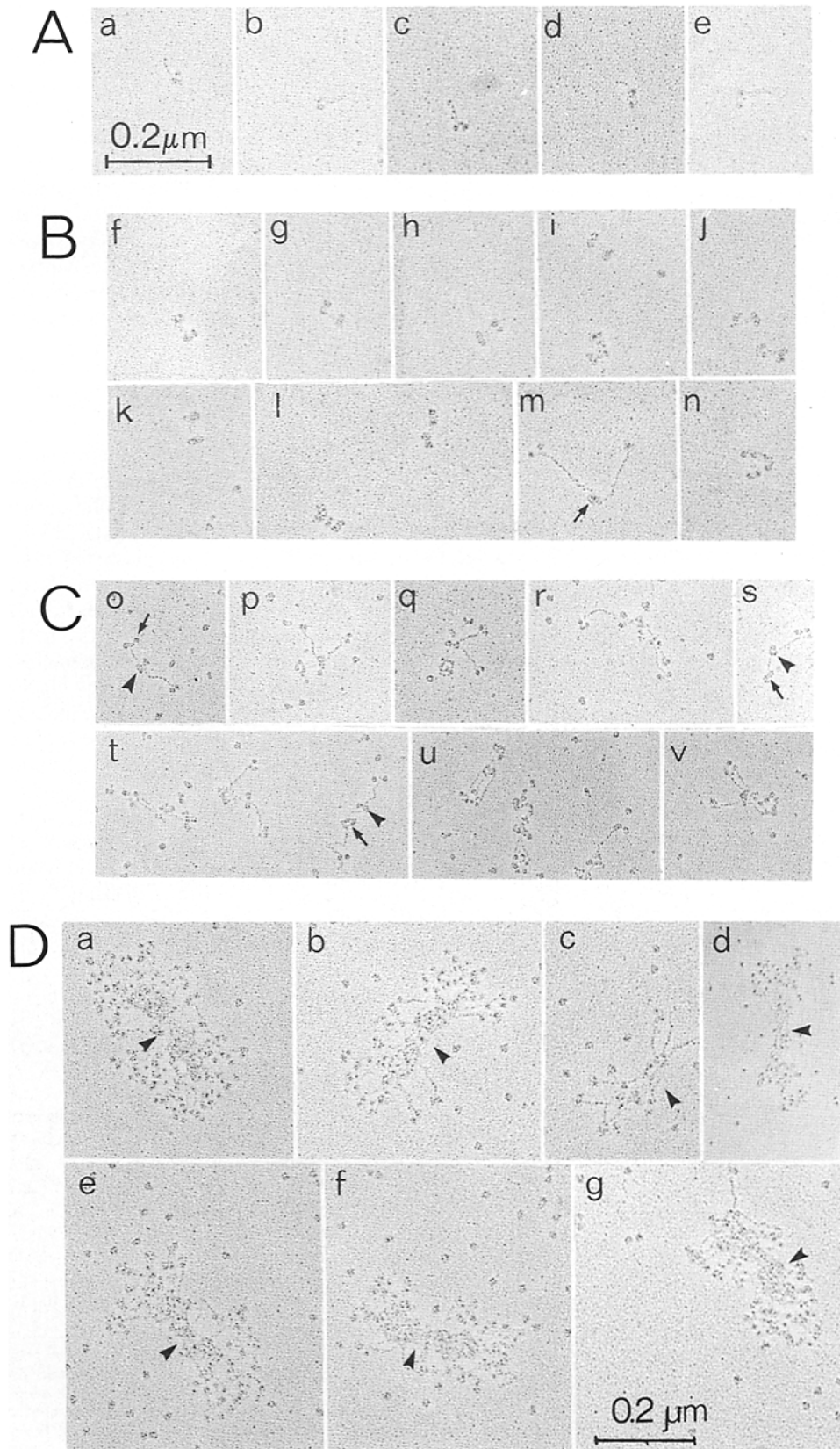


Figure 2. Gallery of selected electron micrographs showing the effects of BM4, BM4 + BM1, and BM1 on the conformation of brush border myosin molecules. (A) Folded brush border myosin molecules, in the absence of antibody. (B) Complexes of myosin with BM4. In *m*, an example of two extended myosin molecules cross-linked by BM4 (arrow) is shown. (C) Complexes of myosin with BM1 and BM4. Arrows and arrowheads in *o*, *s*, and *t* indicate BM4 and BM1, respectively. The myosin molecules shown in *o* and *s* are monomers with an extended tail, present at physiological ionic strength. (D) Complexes of myosin with BM1 (*a-c* and *e-g*), and BM1-Fab (*d*). Central "bare" regions of the filaments (arrowheads). Note the homogeneous size distribution of the filaments. Folded brush border myosin molecules were prepared by dialyzing nonphosphorylated myosin (1 μ M) into a solution containing 175 mM NaCl and 5 mM MgATP (buffer F) and centrifugation at 100,000 *g* (see Materials and Methods). The folded myosin was mixed with \sim 1–2.5 μ M purified monoclonal antibody (or Fab), incubated for 4 h on ice, diluted 1:1 with glycerol, and the mixtures were sprayed immediately onto mica for rotary shadowing.

filament assembly was not because of nonspecific cross-linking of the myosin molecules into aggregates by the bivalent antibody. Thus, when BM1 binds to the folded molecules, the myosin unfolds and the extended molecules can assemble

into short filaments. When examined in electron micrographs after rotary shadowing, these filaments displayed a central zone, apparently devoid of antibodies and myosin heads, \sim 35-nm long and \sim 24-nm wide (Fig. 2 *D*). The two ends

of the filaments were wider (200 ± 50 nm, SD; $n = 68$), more irregular in shape, and contained extended myosin molecules, with their heads oriented away from the central bare zone (Fig. 2 D, arrowheads). When negatively stained and examined in the electron microscope, bipolar filaments with a homogeneous size distribution were observed.

When BM4 was added to the folded molecules, no filaments were observed, and $>90\%$ of the soluble myosin molecules remained in a compact folded conformation, with the antibody bound at the tip of the tail (Fig. 2 B). About a third of the folded molecules were complexed into oligomers by the divalent antibody; i.e., into antiparallel folded myosin dimers with molecules of antibody bound roughly in a central position (Table I) (Fig. 2 B, n).

Finally, we examined electron micrographs of complexes of myosin molecules with BM1 and BM4 together (Fig. 2 C). The feasibility of such an approach was supported by solid phase competition assays, showing that neither antibody inhibited the binding of the other to the same myosin molecule (not shown). Using these two antibodies, one of which (BM1) inhibits folding, while the other (BM4) inhibits assembly, essentially no folded molecules or filaments were observed (Table I). The myosin was present as nonsedimentable species, with extended tails, either as monomers with the two antibodies binding to their respective epitopes (Fig. 2 C, o and s), or as small oligomers cross-linked by one or both antibodies (Fig. 2 C, u and v).

The Antibodies Produce Parallel Changes in Myosin Conformation and Enzymatic Activity

The rate limiting step of myosin ATPase activity is, under most conditions that have been studied, a conformational change that controls the release of the products, ADP and Pi. FTP, a fluorescent ATP analogue, provides a convenient probe to follow the product release steps (Jackson and Bagshaw, 1988a; Cross et al., 1988). In a typical experiment, FTP was first mixed in a slight excess over the myosin active site concentration. When the binding phase was complete, as indicated by the maximum enhancement of fluorescence, the reaction was chased with a >100 -fold molar excess of ATP. The subsequent decrease in fluorescence provides a direct measure of product release and may be described by one or more exponential functions. This approach is particularly powerful as it reveals populations or conformations of myosin, such as the 10S state, which have low turnover rates and may go undetected in conventional steady-state assays.

In the sedimentation and electron microscope studies described above, the nucleotide concentration used to promote the 10S state was ≥ 1 mM ATP and resulted in extensive solubilization of filamentous myosin at low ionic strength. The fluorescence measurements using FTP, however, involved only a low initial nucleotide concentration ($2 \mu\text{M}$) that was insufficient to cause extensive solubilization of preformed filaments. However, if the FTP was added to myosin in 0.6 M NaCl (i.e., initially in the 6S state) and then quickly diluted to 75 mM NaCl, the mixture remained nonturbid and a greater proportion of the FTP became trapped in the 10S form, which released products slowly. Fig. 3 compares the turnover of FTP in 300 mM NaCl, where the predominant state is monomeric 6S myosin, with turnover in 75 mM NaCl, where a mixture of filamentous and 10S myosin

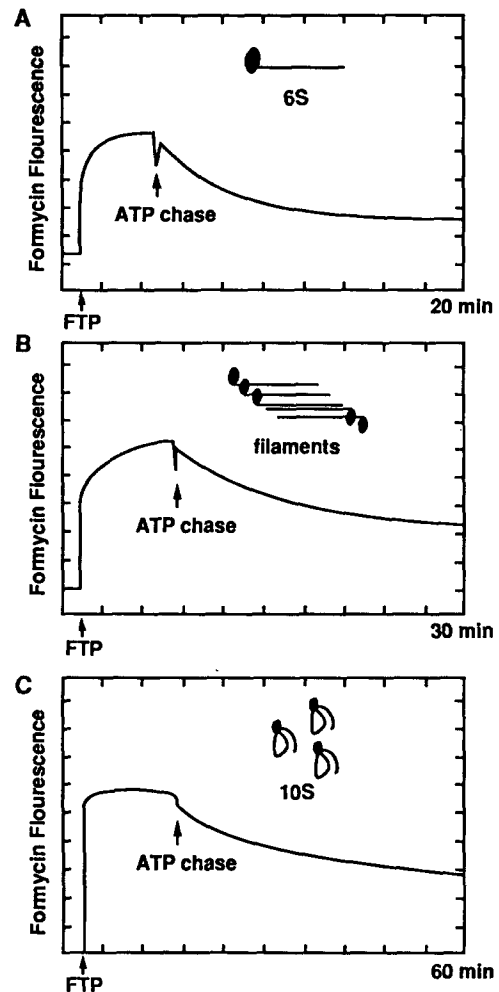


Figure 3. FTP turnover by the different conformational states of brush border myosin. $1 \mu\text{M}$ brush border myosin "heads" were mixed with $2 \mu\text{M}$ FTP, and, when the peak of fluorescence was reached, the reaction was chased with 1 mM MgATP. Reaction conditions: 10 mM imidazole, 1 mM MgCl_2 , 0.5 mM EGTA, 0.5 mM DDT at pH 7.0 and 20°C . (A) 300 mM NaCl, or (B and C) 75 mM NaCl. Formycin fluorescence was monitored using the same fluorimeter settings throughout and the baseline of the plot represents zero light intensity. (A) The myosin was predominantly in the 6S form. A monophasic exponential fit to the fluorescence decay yielded a rate constant of 0.0051 s^{-1} . (B) The myosin was predominantly in the filamentous form and remained in this state when the FTP was added. A biphasic fit yielded rate constants of 0.0013 s^{-1} (80% amplitude) and 0.008 s^{-1} (20% amplitude). (C) The myosin and FTP were premixed in 300 mM NaCl, and then diluted to 75 mM NaCl after 20 s and transferred to the fluorimeter cell. A substantial fraction of the myosin became trapped in the 10S form as judged by the biphasic exponential fit to the data recorded over a 60 -min time base, which yielded rate constants of 0.00066 s^{-1} (90% amplitude) and 0.0062 s^{-1} (10% amplitude).

exists, in proportions dependent on the order of addition of FTP relative to the dilution step. The salt concentration was reduced to 75 mM to ensure that almost all the myosin was in filament form under the conditions used in Fig. 3 B. Analysis of these profiles indicates that the turnover rate for 6S myosin, 10S myosin and filamentous myosin are $5 \times 10^{-3} \text{ s}^{-1}$, $6 \times 10^{-4} \text{ s}^{-1}$, and $1.3 \times 10^{-3} \text{ s}^{-1}$, respectively. The value of the rate constant for the 6S state, however, contains an ele-

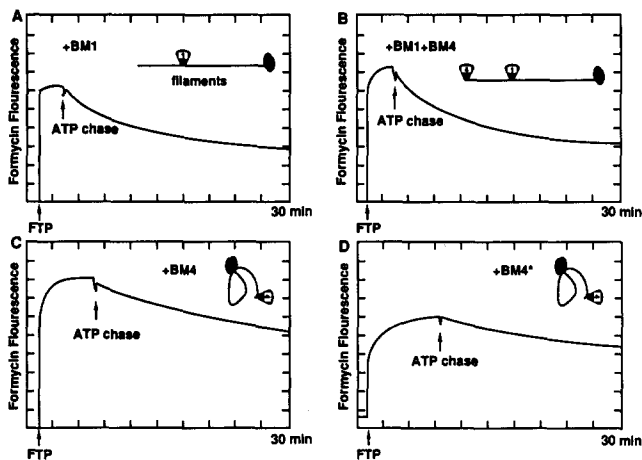


Figure 4. FTP turnover by brush border myosin in the presence of monoclonal antibodies. The fluorimeter settings and reaction conditions were the same as described in Fig. 3. All assays were performed in the final (NaCl) of 75 mM. The antibodies (1–2 μ M) were preincubated with the brush border myosin (0.5 μ M) for 10 min at 20°C, and then a further 0.5 to 1.5 h at 0°C at a (NaCl) of \sim 300 mM. FTP was added and then the mixture diluted after 20 s to 75 mM NaCl, except in *D* where the myosin-antibody mixture was diluted before FTP addition. (*A*) BM1 antibody that resulted in filamentous myosin. A biphasic exponential fit yielded rate constants of 0.0012 s^{-1} (78% amplitude) and 0.0061 s^{-1} (22% amplitude). (*B*) BM1 + BM4 antibody that held the myosin in the 6S conformation. A biphasic fit yielded rate constants of 0.0017 s^{-1} (76% amplitude) and 0.097 s^{-1} (24% amplitude). (*C*) BM4 antibody that held the myosin in the 10S conformation. A biphasic fit yielded rate constants of 0.00046 s^{-1} (92% amplitude) and 0.0031 s^{-1} (8% amplitude). (*D*) As above but the myosin was diluted to 75 mM NaCl before FTP addition. A biphasic exponential fit yielded rate constants of 0.00053 s^{-1} (94% amplitude) and 0.0083 s^{-1} (6% amplitude). Records (*C*) and (*D*) were allowed to proceed for 60 min to give an adequate fit to the slow phase.

ment arising from the higher ionic strength. Using monoclonal antibodies to hold the myosin in a defined conformation potentially allows this problem to be overcome.

FTP turnover by myosin preparations that had been preincubated with monoclonal antibodies are shown in Fig. 4. BM1 alone prevents the formation of the 10S state, and, in its presence, myosin shows a moderately high FTP turnover rate ($1.2 \times 10^{-3} s^{-1}$) (Fig. 4 *A*) comparable with the filamentous form (Fig. 3 *B*). The enzymatic activity is further increased in the presence of both BM1 and BM4, and this condition permits a measurement of the turnover rate ($1.6 \times 10^{-3} s^{-1}$) of the 6S conformation at low ionic strength (Fig. 4 *B*). In contrast, BM4 alone prevents filament assembly and on dilution to low ionic strength formation of the 10S state is promoted. This is evident from the very slow FTP turnover rate ($4.5 \times 10^{-4} s^{-1}$) under these conditions (Fig. 4 *C*). Furthermore the 10S state predominates regardless of the order of addition of FTP relative to dilution. Indeed when the BM4-myosin mixture was diluted to 75 mM NaCl before FTP addition (Fig. 4 *D*), the solution remained clear, indicative of the myosin being fixed in a monomeric state (cf., Fig. 1 *C*). As soon as the FTP binds under these conditions, the myosin must rapidly fold into the 10S state and trap the products. This contrasts with the corresponding experiment in the

absence of BM4 (Fig. 3 *B*) where the filamentous myosin turns over the FTP relatively rapidly before the filaments can depolymerize, and only a small fraction of the myosin can trap the formycin nucleotide before the FTP is exhausted or is competed out with ATP.

To confirm these effects on the antibodies on myosin filament assembly, aliquots of the FTPase assay solutions at the end of the experiments were analyzed in the electron microscope after negative staining. No filaments were observed in the myosin samples in the presence of BM4 or in the presence of BM1 + BM4, whereas in the control and BM1 preparations, filaments with approximately uniform dimensions were clearly visible (Fig. 5). The control sedimentation experiments shown in Fig. 1 *C* confirm that at 75 mM NaCl, MgATP has very little effect on preformed filaments (only \sim 15% of the myosin is soluble); whereas, when added to the myosin in high salt before dilution to 75 mM NaCl, at least 65% of the myosin then remains soluble. Otherwise, the effects of the antibodies on the “solubility” of the myosin at 75 mM salt are qualitatively similar to those observed at about physiological ionic strength (175 mM NaCl) (compare Figs. 1, *B* and *C*).

Discussion

Monoclonal Antibodies Modulate Myosin Monomer-Polymer Equilibria by Locking Myosin into One Conformational State

We have described a novel approach to investigate the influence of myosin conformation on its enzymatic activity, using specific monoclonal antibodies. Antibodies BM4 and BM1 alone lock the myosin into either a monomer or filamentous state, and, when used in combination, induce the myosin molecule to be in an extended monomeric conformation, under conditions where normally it would be either in a folded conformation or in filaments. Such specific effects on myosin conformation have not been described in previous studies using monoclonal antibodies to invertebrate myosins and vertebrate smooth and skeletal muscle myosins, although effects on filament formation and the actin-activated myosin ATPase activity were observed (Kiehart et al., 1984; Kiehart and Pollard, 1984; Schneider et al., 1985; Winkelmann and Lowey, 1986; Pagh and Gerisch, 1986; Citi and Kendrick-Jones, 1988).

BM4 shifts the monomer-polymer equilibrium of brush border myosin towards the monomeric, unassembled state. As suggested previously (Citi and Kendrick-Jones, 1988), BM4, by binding to the tip of the myosin tail, inhibits the antiparallel interaction occurring in the initial step of filament assembly, and therefore blocks nucleation. BM4, however, does not inhibit the folding of brush border myosin or unfold previously folded molecules, showing that the tip of the rod can freely interact with the antibody without interfering with the folding process.

BM1 allows nucleation to occur but hinders elongation; thus the filaments formed are shorter (Citi and Kendrick-Jones, 1988) (Fig. 5). This antibody inhibits the transition between extended (6S) and folded (10S) myosin molecules (at physiological ionic strength and in the presence of MgATP) (Fig. 1 and 2), and “locks” the myosin molecules in the extended state. These extended monomers can then as-

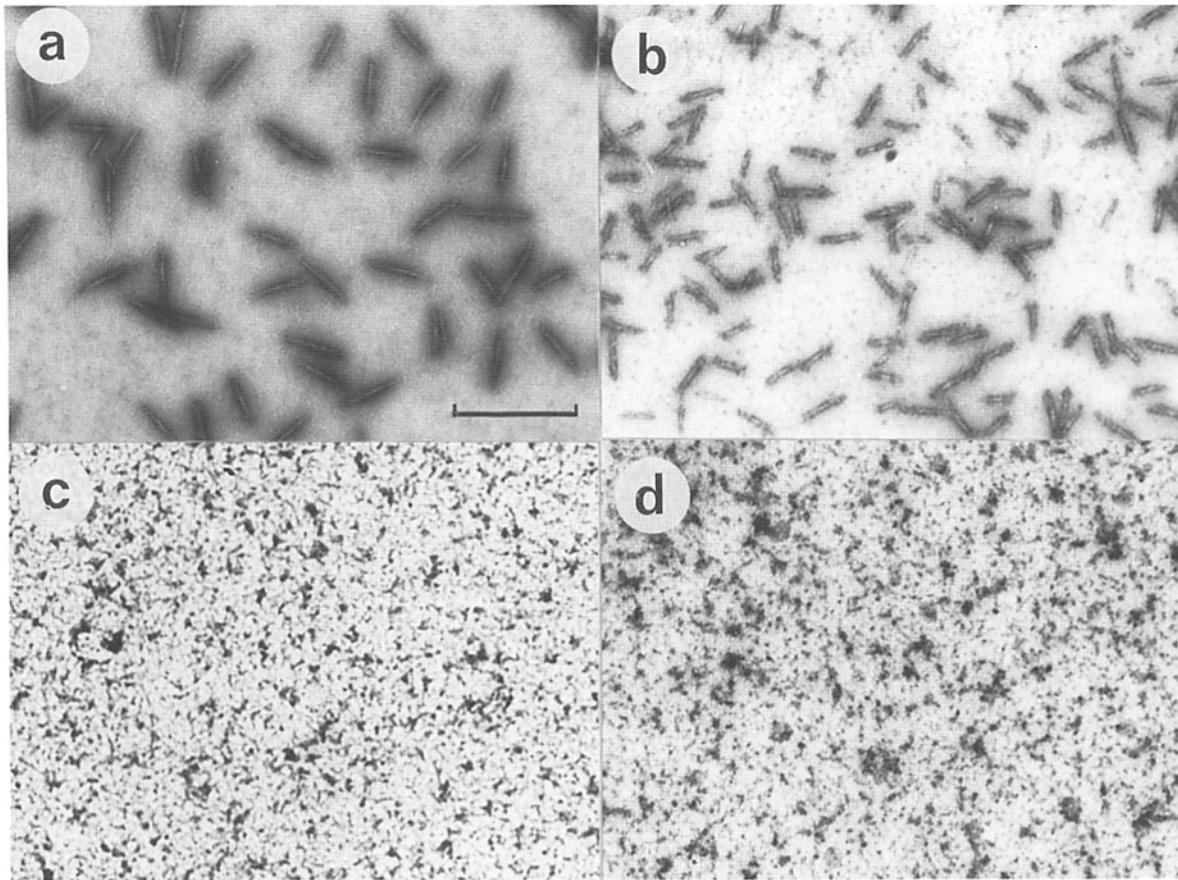


Figure 5. Electron microscopic analysis of the effects of antibody binding on the assembly of brush border myosin in samples taken from the FTPase assays. (a) Brush border myosin filaments in the absence of antibody; (b) short myosin filaments in the presence of BM1; (c) the effects of BM4; and (d) BM1 + BM4 on myosin filament assembly. Note the absence of recognisable filaments in c and d, instead an amorphous background of protein. For negative staining and electron microscopy, samples were taken directly from the assay solutions at the end of the experiments shown in Fig. 4 (conditions: 1 μM myosin heads and 1–2 μM antibodies in 75 mM NaCl, 10 mM imidazole, pH 7.0, 0.5 mM EGTA, 1 mM MgCl_2 , 0.5 mM DTT plus 2 μM FTP and 1 mM MgATP).

semble into filaments via the antiparallel interactions of the myosin tails. There are a number of possible ways to explain how this antibody prevents the myosin tail from folding. Firstly, if charge interactions between segments of the tail are involved in stabilizing the folded conformation (Trybus and Lowey, 1984), it is possible that BM1 could inhibit such interactions, possibly by altering the charge distribution on the surface of the tail or by physically preventing the tail segments from interacting. On the other hand, the folded conformation could be stabilized by an interaction between the neck region of the myosin head and the tail, near its proximal hinge region (Craig et al., 1983). Since this region on the tail is close to the epitope where BM1 binds, BM1 may sterically inhibit such interaction. Thirdly, since BM1 binds near to one of the flexible sites in the myosin tail involved in folding, it could modify the conformation of this region by locally perturbing the structure of the α helical coiled coil. Finally, we cannot exclude the possibility that the antibody may act via some yet unidentified long-range effect on the conformation of the whole molecule.

Enzymology of the 6S Myosin Monomer-BM1-BM4 Complex at Low Ionic Strength and Its Relevance In Vivo

The extended tail monomer conformation (6S) of brush border myosin is unstable at physiological ionic strength. By stabilizing this energetically unfavorable conformation in vitro, BM1 and BM4 facilitate investigation of its enzymology. This 6S-myosin monomer-antibody complex has an FTPase activity very similar to that of the molecules assembled into filaments. Thus, when folding of the myosin tail is prevented by these antibodies, trapping of nucleotide does not occur. This indicates that the trapping of nucleotide at the active site is not because of a change in the structure of the myosin head, occurring at low ionic strength, but because of the folding of the myosin tail. Thus, filamentous myosin and the 6S-myosin-BM1 + BM4 complex at low ionic strength and the 6S-myosin at high ionic strength all have similar FTPase rates. In the 10S myosin-BM4 complex, the antibody maintains the folded state, and so the FTPase activity is low.

Studies are in progress to determine the enzymatic activity of the 6S myosin-antibody complex (myosin-BM1 + BM4) in the presence of actin *in vitro*. These studies require the use of monovalent antibody fragments, since in the presence of actin and the two bivalent antibodies the myosin shows an increased tendency to form oligomers and aggregates (Citi and Kendrick-Jones, unpublished observations). We have previously shown that in phosphorylated myosin, there is a correlation between the extent to which these antibodies block filament assembly and the degree to which they inhibit the steady-state actin-activated MgATPase of the myosin (Citi and Kendrick-Jones, 1988). BM4-Fab was found to be the most potent inhibitor of the myosin actin-activated MgATPase (Citi and Kendrick-Jones, 1988), because not only does it prevent filament assembly, but it also appears to be the most effective at preventing aggregation which tends to occur in the presence of actin.

BM1 and BM4 have also been used in microinjection studies to probe myosin function *in vivo* (Hoener et al., 1988). When these antibodies were separately injected into fibroblasts, they increased cell motility and membrane dynamics, and produced bizarre cell shapes, suggesting that they interfere with myosin interaction with actin probably by reducing the amount of functional myosin in the cell (Hoener et al., 1988). Since we envisage that this is because of the ability of the antibodies to lock the myosin into filamentous (BM1) or monomer (BM4) conformations, we suggest that the dynamic rearrangements and transitions between the folded and filamentous states of the myosin are critical for its function within the cell.

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