Subunit Exchange between Smooth Muscle Myosin Filaments

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Abstract. Filaments formed from phosphorylated smooth muscle myosin are stable in the presence of MgATP, whereas dephosphorylated filaments are disassembled to a mixture of folded monomers and dimers. The stability of copolymers of phosphorylated and dephosphorylated myosin was, however, unknown. Gel filtration, sedimentation velocity, and pelleting assays were used to show that MgATP could dissociate dephosphorylated myosin from copolymers containing either rod and myosin or dephosphorylated and phosphorylated myosin. Copolymers were typically formed by dialyzing monomeric mixtures into filament-forming buffer but, unexpectedly, could also be formed within minutes of mixing preformed rod and myosin minifilaments. This result suggested that molecules can rapidly and extensively exchange between filaments, presumably via the monomeric pool of myosin in equilibrium with polymer. An exchange of molecules between filaments was demonstrated directly by electron microscopy using gold-labeled streptavidin or antibody to detect the exchanged species. By this approach it was shown that smooth muscle myosin filaments, like other macromolecular assemblies, are dynamic structures that can readily alter their composition in response to changing solvent conditions. Moreover, because folded monomeric myosin is unable to polymerize, these experiments suggest a mechanism for the disassembly of the filament by MgATP.

THE observation that dephosphorylated smooth muscle myosin filaments in vitro are rapidly disassembled by MgATP (Suzuki et al., 1978) raised the question of the state of assembly of myosin in a smooth muscle cell. Rapid freezing techniques, in conjunction with measurements of the state of light chain phosphorylation, showed that myosin that was predominantly dephosphorylated could exist in the filamentous form in situ (Somlyo et al., 1981). In order to reconcile these two observations, it would appear that filaments must be stabilized in some way in vivo. Possible ways that can be envisioned are by binding of yet unidentified accessory proteins, by virtue of the exact ionic conditions and protein concentrations in the cell, or by the presence of a small amount of phosphorylated myosin in each filament.

To test the extent to which phosphorylated myosin could stabilize a copolymer, MgATP was added to minifilaments formed from monomeric mixtures of phosphorylated and dephosphorylated myosin (see the preceding article for a description of this model filamentous system). Unexpectedly, dephosphorylated myosin disassembled from the copolymer despite the presence of the more stable neighboring phosphorylated molecules. Not only were phosphorylated molecules unable to stabilize the filament as a whole, but molecules in the filament were shown to rapidly exchange with molecules in the monomer pool. Within minutes of mixing homopolymers of phosphorylated and dephosphorylated myosin, copolymers were formed. An exchange of molecules in and out of the filament provides a mechanism by which dephosphorylated myosin could disassemble even from predominantly phosphorylated copolymers upon addition of nucleotide. The free monomeric form of smooth muscle myosin adopts a stable, folded conformation and as a consequence is unable to reenter the polymer (Cross et al., 1986).

Even though skeletal filaments are considered to be less labile than smooth muscle myosin filaments, exchange of myosin molecules between filaments was first observed in this system. Using a fluorescence energy-transfer assay, Saad et al. (1986) recently showed extensive exchange between the soluble pool of myosin and skeletal muscle myosin filaments with a half-time <10 min. Compared with these limited studies on myosin, the exchange properties of other cytoskeletal proteins such as tubulin and actin have received considerably more attention over the past few years. Even here, however, it is not clear which of the various proposed exchange mechanisms will ultimately be shown to occur in vivo. End exchange of monomers, directional exchange or treadmilling of monomers (Wegner, 1976; Margolis and Wilson, 1978), or rapid assembly and disassembly of individual polymers ("dynamic instability" model, Kirschner and Mitchison, 1986) are all possible ways for subunit exchange to occur. The assembly of myosin into filaments differs from the polymerization of actin and tubulin in that nucleotide is not required for assembly. Nevertheless, from the experiments described here as well as those with skeletal myosin (Saad et al., 1986), it appears that even macromolecular assemblies that were considered relatively static, could be undergoing a constant turnover of molecules.

Materials and Methods

Protein Preparation

Turkey gizzard myosin was prepared by the method of Sellers et al. (1981). Myosin in 0.2 M ammonium acetate, pH 7.2, was digested to subfragment-1 and rod with papain. The rod fraction was purified by ethanol fractionation (Margossian and Lowey, 1982). Protein concentrations were determined using $E^{1\%}_{280} = 5.0$ for myosin and $E^{1\%}_{280} = 0.86$ for rod. Polyclonal antibody against chicken pectoralis myosin was isolated and affinity purified as described in Gauthier and Lowey (1979).

Gel Filtration

A 1.6 × 60-cm column equipped with flow adaptors was packed with Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). The column was equilibrated in 5 mM citrate/22 mM Tris, 1 mM MgATP, 0.1 mM EGTA, 1 mM NaN_3. 1 mM MgATP and 0.1 mM EGTA were added to 0.9 ml of 3.5 mg/ml minifilaments (5 mM citrate/22 mM Tris) just before sample application. Flow rate was 16 ml/h, and 1.45-ml fractions were collected. The column profile was determined by fluorescence measurements (MPF 44 fluorimeter, Perkin-Elmer Corp., Norwalk, CT; excitation wavelength 295 nm, emission wavelength 330 nm).

Centrifugation

Sedimentation velocity measurements were carried out in a model E analytical ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA), using the schlieren optical system.

Pelleting Assay for Copolymer Formation

An airfuge (Beckman Instruments, Inc.) operated at 23 psi (130,000 *g*) for 10 min was used to pellet actin–minifilament complexes. Under these conditions, none of the rod minifilaments and approximately half of the myosin minifilaments sedimented. Upon addition of actin, rod minifilaments remained in the supernatant, but myosin minifilaments were completely pelleted. The appearance of rod in the pellet is therefore a measure of copolymer formation when preformed rod and myosin minifilaments are mixed. Rod and myosin minifilaments (0.5 mg/ml each) were incubated for varying lengths of time; 1.5 mg/ml actin was then added. The final buffer composition was 5 mM citrate/22 mM Tris, 5 mM KCl, 1 mM $MgCl_2$. The KCl and $MgCl_2$ were needed to decrease the amount of monomeric actin whose critical concentration in citrate/Tris alone was ~0.5 mg/ml.

Filament-pelleting Assay

Varying ratios of phosphorylated and dephosphorylated myosin (3 mg/ml total) were dialyzed into 5 mM citrate/16 mM Tris, 1 mM EGTA, then into this same buffer that also contained 0.1 M KCl and 7 mM $MgCl_2$ for synthetic filament formation. 1 mM MgATP was added and the filaments pelleted in an airfuge (23 psi, 130,000 *g*, 10 min). Protein concentrations were determined by the Bradford method (1976) with a myosin standard curve. The degree of light chain phosphorylation was determined by glycerol/acrylamide gels (Perrie and Perry, 1970).

Platinum Shadowing of Copolymers

Myosin minifilaments (1.25 mg/ml in 5 mM citrate/22 mM Tris) were incubated with rod minifilaments (0.5 mg/ml) for 10 min, and then cross-linked (at 1 mg/ml total protein) for 15 min on ice with 1.5 mg/ml dimethylsuberimidate. The cross-linking reaction was stopped by removal of the reagent on a centrifuged G-50 column (Neal and Florini, 1973). The samples were then diluted to 20 μg/ml in 5 mM pyrophosphate/66% glycerol for platinum shadowing by the method of Tyler and Branton (1980). Electron micrographs were taken on a Philips EM 301 microscope operated at 60 kV.

Exchange of Skeletal Myosin into Smooth Muscle Myosin Filaments by Electron Microscopy

Filaments were formed from turkey gizzard myosin and chicken pectoralis myosin by dialyzing first into 5 mM citrate/16 mM Tris, then into 5 mM citrate/16 mM Tris, 0.1 M KCl. In control experiments, 30 μg/ml filaments (5 μl) were incubated with 100 μg/ml polyclonal anti-chicken pectoralis myosin IgG (5 μl) for 2 min; goat anti-rabbit IgG conjugated to 10-nm gold particles (5 μl, Janssen Pharmaceutica, Beerse, Belgium; G10 EM grade, stock diluted 1.4-fold) was then added and the mixture was incubated for another 2 min. This mixture (15 μl) was put onto a UV-irradiated carbon-formvar grid for 30 s, washed with 10 drops of filament buffer, and stained with 5 drops of 1% uranyl acetate. The last drop was wicked off and the grids were air-dried at room temperature. For following the incorporation of skeletal myosin into smooth muscle myosin filaments, chicken pectoralis myosin filaments were pelleted in the Beckman Instruments, Inc. airfuge (23 psi, 130,000 *g*, 10 min) to obtain the monomer pool of myosin in the supernatant. This critical concentration of skeletal myosin was incubated with 30–300 μg/ml turkey gizzard myosin filaments overnight on ice. Incubation with antibodies and negative staining are as described above.

Biotinylation of Myosin and Microscopy of Streptavidin-Gold–labeled Filaments

Myosin (2 mg/ml) in 10 mM KP_i, pH 8, 0.1 M KCl, 5 mM $MgCl_2$ was biotinylated with 16–40 μM *N*-hydroxysuccinimido biotin (NHS-biotin, Sigma Chemical Co., St. Louis, MO) (2 mM stock in dimethylsulfoxide) overnight on ice or 60 min at room temperature. The reaction was stopped by the addition of 5 mM glycine/Tris, pH 8, followed by removal of excess reagents on a centrifuged G-50 column equilibrated in high salt (Neal and Florini, 1973) or by dialysis against high-salt buffer. This reagent was used to modify the myosin used in the experiment shown in Fig. 7. NHS-LC-biotin (sulfosuccinimidyl 6-[biotinamido] hexanoate, Pierce Chemical Co., Rockford, IL), a water-soluble, long-chain analogue of *N*-hydroxysuccinimido biotin, was used to modify the myosin used in the experiment shown in Fig. 8. 2 mg/ml myosin in 10 mM KP_i, pH 8, 0.1 M KCl, 5 mM $MgCl_2$ was reacted with 50 μM NHS-LC-biotin overnight on ice. The reaction was stopped as described above. This reagent did not seem to affect the ability of the myosin to assemble into filaments in 10 mM imidazole, pH 7, 0.1 M KCl, 5 mM $MgCl_2$. Alternatively, 2 mg/ml myosin in 10 mM Tris, pH 8.5 (4°C), 50 mM KCl was reacted overnight on ice with 5–10 mM *N*-iodoacetyl-*N'*-biotinylhexylenediamine (Pierce Chemical Co.) that was added as a solid. The reaction was stopped by addition of 20 mM dithiothreitol. This reagent gave a lower degree of modification than the lysine-specific reagents, but similar results were obtained when this myosin was used in exchange experiments. The biotinylation reaction was followed by a modified ELISA. Serial dilutions of biotinylated myosin samples or biotinylated IgG, which was used as a standard, were bound to a microtiter plate. Streptavidin-β-galactosidase (500-fold diluted) was then added followed by 1 mg/ml *p*-nitrophenyl-β-D-galactopyranoside. Absorbance at 405 nm was read on an EL 308 plate reader (Bio-Tek Instruments, Inc., Burlington, VT).

Exchange of myosin molecules between filaments was followed in different solvents and at different total protein concentrations. In Fig. 7, equal concentrations of turkey gizzard myosin filaments and filaments biotinylated with NHS-biotin (50 μg/ml total protein concentration, 5 mM citrate/16 mM Tris, 0.1 M KCl) were incubated together for 2–60 min. In Fig. 8, NHS-LC-biotin was used to modify the myosin; the filaments were incubated for longer times (24–72 h) at higher protein concentrations (1.3 mg/ml) and in a different solvent (10 mM imidazole, pH 7, 0.1 M KCl, 5 mM $MgCl_2$) than in Fig. 7. In either case, 10 μl of streptavidin-colloidal gold (Bethesda Research Laboratories, Gaithersburg, MD; 20 nm gold, twofold diluted) were added to 10 μl of 50 μg/ml filaments, and the mixture was incubated for 30 s in solution. This solution was then placed on the grid and negatively stained as described in the previous section.

Results

MgATP Dissociates Dephosphorylated Myosin from Copolymers

Dephosphorylated minifilaments, small bipolar aggregates composed of 12–14 molecules (5 mM citrate/22 mM Tris), are dissociated by MgATP to a mixture of folded monomers and antiparallel folded dimers; phosphorylated minifilaments remain assembled (see preceding article). Although this property of homopolymers is well established, the stability of copolymers was not known. To determine whether dephosphorylated myosin can be stabilized in the filamentous form by phosphorylated myosin, varying ratios of the two

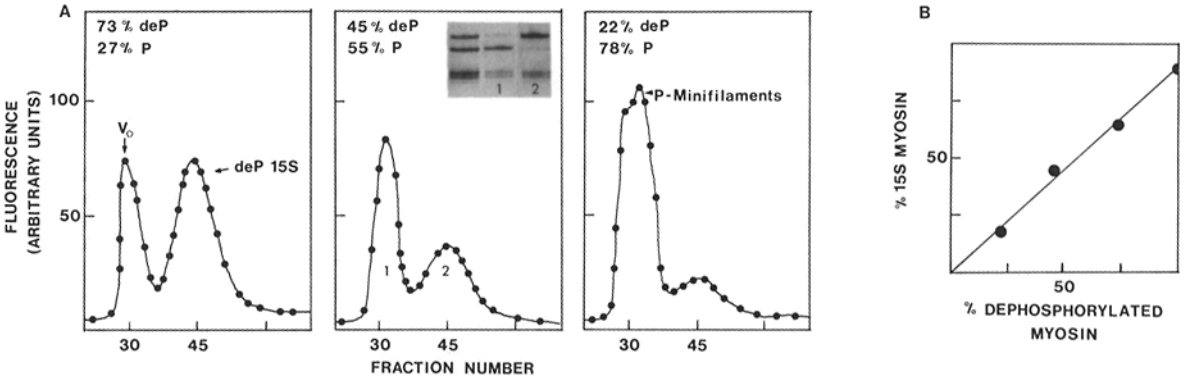


Figure 1. MgATP dissociates dephosphorylated myosin from copolymers. (*A*) Copolymers were formed from mixtures containing ~25%, 50%, or 75% phosphorylated myosin. 1 mM MgATP was added, and minifilaments (void volume, first peak) were separated from the 15S species by gel filtration on Sepharose 4B. (*Inset*) Gel shows the state of phosphorylation of the starting material and the separated species. From top to bottom, the bands are dephosphorylated 20-kD light chain, phosphorylated 20-kD light chain, and 17-kD light chain. (*B*) The percent disassembled myosin is plotted as a function of the percent dephosphorylated myosin in the copolymer.

species were mixed in high salt, and dialyzed into citrate/Tris for minifilament formation. Upon addition of 1 mM MgATP to these copolymers, both the folded and the filamentous form of myosin were observed by sedimentation velocity. Centrifugation could not be used to quantitate the relative amounts of the 22S minifilament and the 15S folded myosin because of the similarity of their sedimentation coefficients. Because the shapes of the species are so different, however, gel filtration could be used to separate filamentous from folded myosin (Trybus and Lowey, 1985) (Fig. 1 *A*). The data indicate that essentially all the dephosphorylated myosin in the copolymer disassembled to the 15S folded form in the presence of MgATP, even when the copolymer contained more than half phosphorylated myosin (Fig. 1 *B*). A possible explanation for this result was that the two myosin species did not copolymerize. In that the same minifilament is formed in the absence of MgATP regardless of the state of light chain phosphorylation, there is no way to demonstrate copolymer formation.

The ability of myosin species to copolymerize was therefore investigated by using myosin and rod, the proteolytic fragment of myosin without the head. Rod forms a minifilament type of structure that, like phosphorylated myosin, remains assembled in the presence of MgATP. The sedimentation coefficient of rod minifilaments ($s°_{20,w}$ = 13S), however, is approximately half that of myosin minifilaments ($s°_{20,w}$ = 22S) (see preceding article). Copolymer formation between rod and myosin can therefore be detected by a change in sedimentation rate from that of the homopolymers, as shown in Fig. 2, *A–C*. A single boundary of intermediate mobility (14S) was obtained when an equimolar mixture of rod and myosin was dialyzed from high salt into citrate/Tris. When MgATP was added to this known copolymer, dephosphorylated myosin depolymerized as usual to its favored folded conformation, as evidenced by gel filtration (data not shown). Thus, even the more stable rod molecules cannot maintain dephosphorylated myosin in the filamentous state in the presence of nucleotide.

Minifilaments Rapidly Exchange Molecules

A possible explanation for this lack of stabilization of dephosphorylated myosin by the rod was provided by the following experiments. When rod minifilaments and myosin minifilaments were sedimented in the same cell in the analytical ultracentrifuge, only a single boundary, and not the expected two boundaries, was observed. The single boundary (Fig. 2 *D*) sedimented at a rate similar to that observed when copolymers were formed from monomeric mixtures in high salt (Fig. 2 *C*). One way that two populations of homopolymers can form copolymers is for molecules to dissociate from the filament, enter the monomer pool, and then randomly reassociate with either homopolymer until all filaments contain both types of molecules. An unexpected feature of the single boundary was that it formed within 15 min of mixing the homopolymers.

The time course of copolymer formation could be followed more precisely by a pelleting assay. Rod minifilaments were incubated with myosin filaments for 2–40 min; actin was then added and the mixture was pelleted in the airfuge. Myosin binds to actin and is pelleted regardless of the time of in-

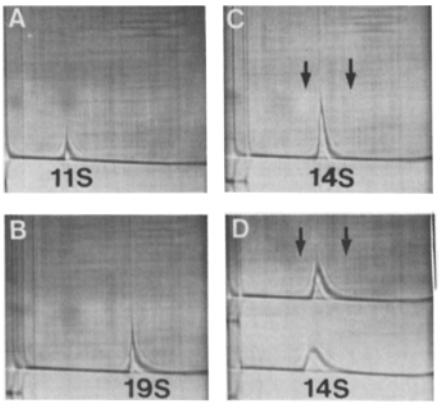


Figure 2. Copolymers are formed from mixtures of homopolymers. Sedimentation patterns of (*A*) rod minifilaments, (*B*) myosin minifilaments, and (*C*) the copolymer formed from an equimolar mixture of rod and myosin. (*D*) The patterns obtained 4 h (*upper*) or 15 min (*lower*) after mixing rod minifilaments with myosin minifilaments. Arrows indicate the position of rod and myosin minifilaments if no exchange had occurred. Protein concentration, 0.44 mg/ml rod, 1 mg/ml myosin.

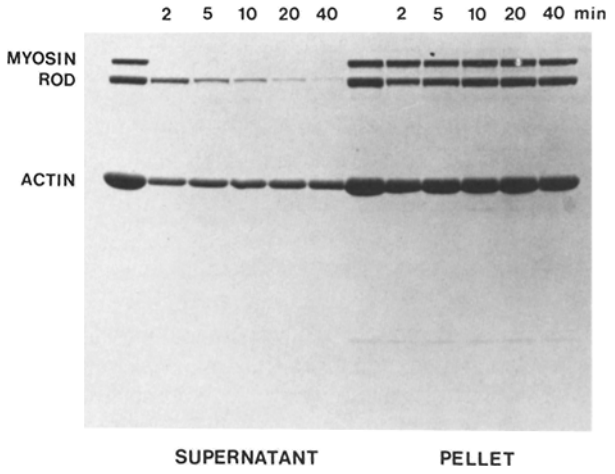


Figure 3. Time course of copolymer formation. Rod and myosin minifilaments (0.5 mg/ml each) were incubated for the indicated length of time; 1.5 mg/ml actin was then added and the mixture pelleted in the airfuge. The disappearance of rod in the supernatant and the appearance of rod in the pelleted fraction were followed by SDS-gel electrophoresis.

cubation, but rod will be found in the pellet only if it is present in a filament that also contains myosin. Fig. 3 shows that approximately half of the rod molecules were recovered in the pellet after a 2-min incubation. (Note that an additional 10-min period elapses before the pelleting is complete.) After a 40-min incubation of the filaments, essentially no rod remains in the supernatant. There was no observable difference in the time course if dephosphorylated or phosphorylated myosin was used. This assay confirmed the observation that copolymers are rapidly formed simply by mixing populations of filaments. Metal-shadowed images of the polymers obtained after a 15-min incubation of rod and myosin minifilaments showed directly the presence of both rod and myosin in the same filament (Fig. 4).

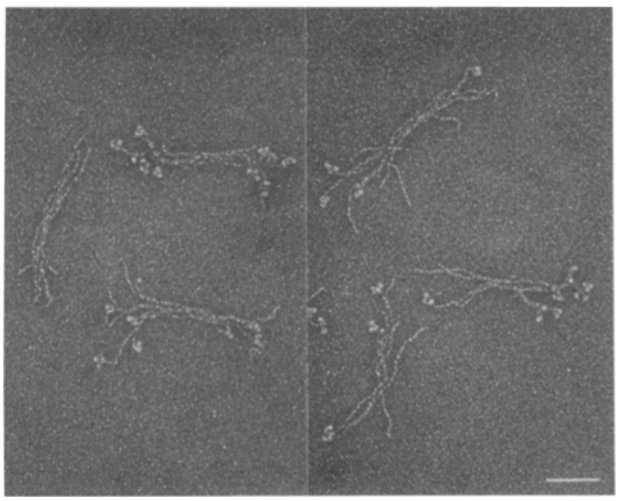
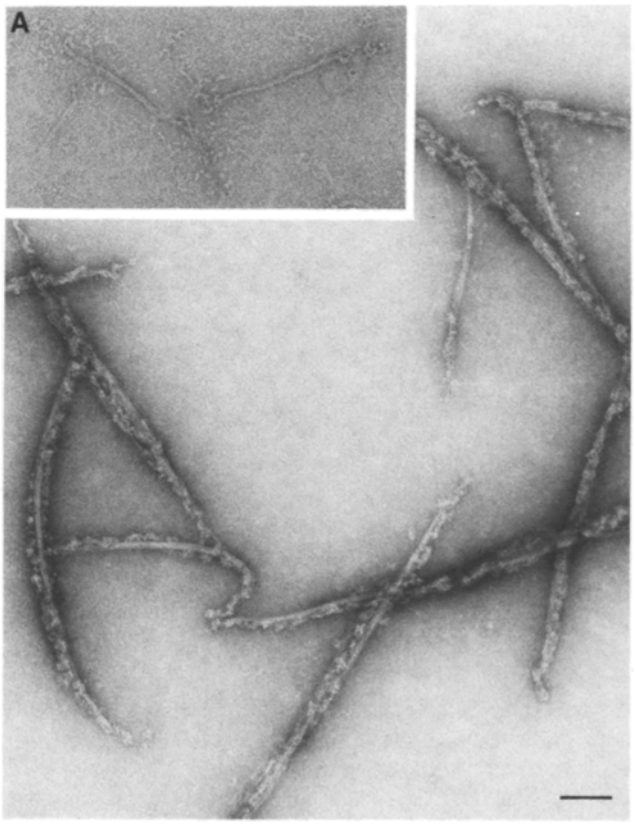


Figure 4. Electron micrograph of metal-shadowed copolymers. Copolymers obtained after a 15-min incubation of rod and myosin minifilaments. Bar, 0.1 μm.



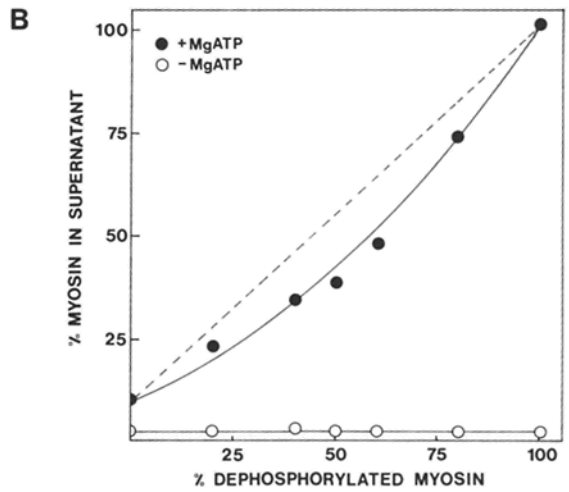


Figure 5. Dephosphorylated myosin is dissociated from larger synthetic filaments. (*A*) Synthetic filaments were formed by dialyzing minifilaments (inset) containing varying ratios of dephosphorylated myosin into 5 mM citrate/22 mM Tris, 0.1 M KCl, 7 mM $MgCl_2$. Bar, 0.1 μm. (*B*) 1 mM MgATP was added to these phosphorylated/dephosphorylated copolymers, and the filaments pelleted in the airfuge. The amount of disassembled myosin in the (●) presence or (○) absence of 1 mM MgATP is plotted as a function of the percent dephosphorylated myosin in the copolymer.

Exchange in Larger Synthetic Filaments

In order to determine whether rapid exchange of molecules occurs in larger synthetic filaments, minifilaments were dialyzed into citrate/Tris that contained 0.1 M KCl and 7 mM $MgCl_2$, conditions that cause the filaments to grow in both

length and width (Fig. 5 *A*). Because these large synthetic filaments sediment at a much faster rate than the folded conformation, the polymer could be pelleted without adding actin, and the amount of folded myosin remaining in the supernatant quantitated. In the absence of MgATP, <5% of the myosin is recovered in the supernatant (Fig. 5 B, ○). Upon addition of 1 mM MgATP to copolymers containing the indicated fraction of dephosphorylated myosin, most of the dephosphorylated myosin was solubilized (Fig. 5 B, ●). The deviation of the points from the dashed line indicates that a small amount of dephosphorylated myosin was stabilized in the filamentous form. Nevertheless, the majority of the dephosphorylated molecules disassembled even when present in a copolymer containing an excess of phosphorylated myosin, in agreement with the results obtained with the smaller minifilaments.

This result suggested that, if exchange of molecules is the mechanism whereby the dephosphorylated molecules dissociate from the filament, then this process must include subunits that were initially distributed along the length of the filament. If exchange of molecules with the monomer pool was restricted to the existing filament ends, only a small percentage of the dephosphorylated myosin would be recovered in the supernatant, assuming a random distribution of phosphorylated and dephosphorylated myosin throughout the filament.

Localization of Exchanged Myosin by Electron Microscopy

The exchange of skeletal myosin into smooth muscle myosin filaments was used to demonstrate that molecules can be incorporated along the filament length. Skeletal myosin was detected by incubating the filaments with a primary antibody specific for chicken pectoralis myosin, followed by a secondary antibody (goat anti-rabbit) that was conjugated to gold. Fig. 6 *A* shows that the smooth muscle myosin filaments are

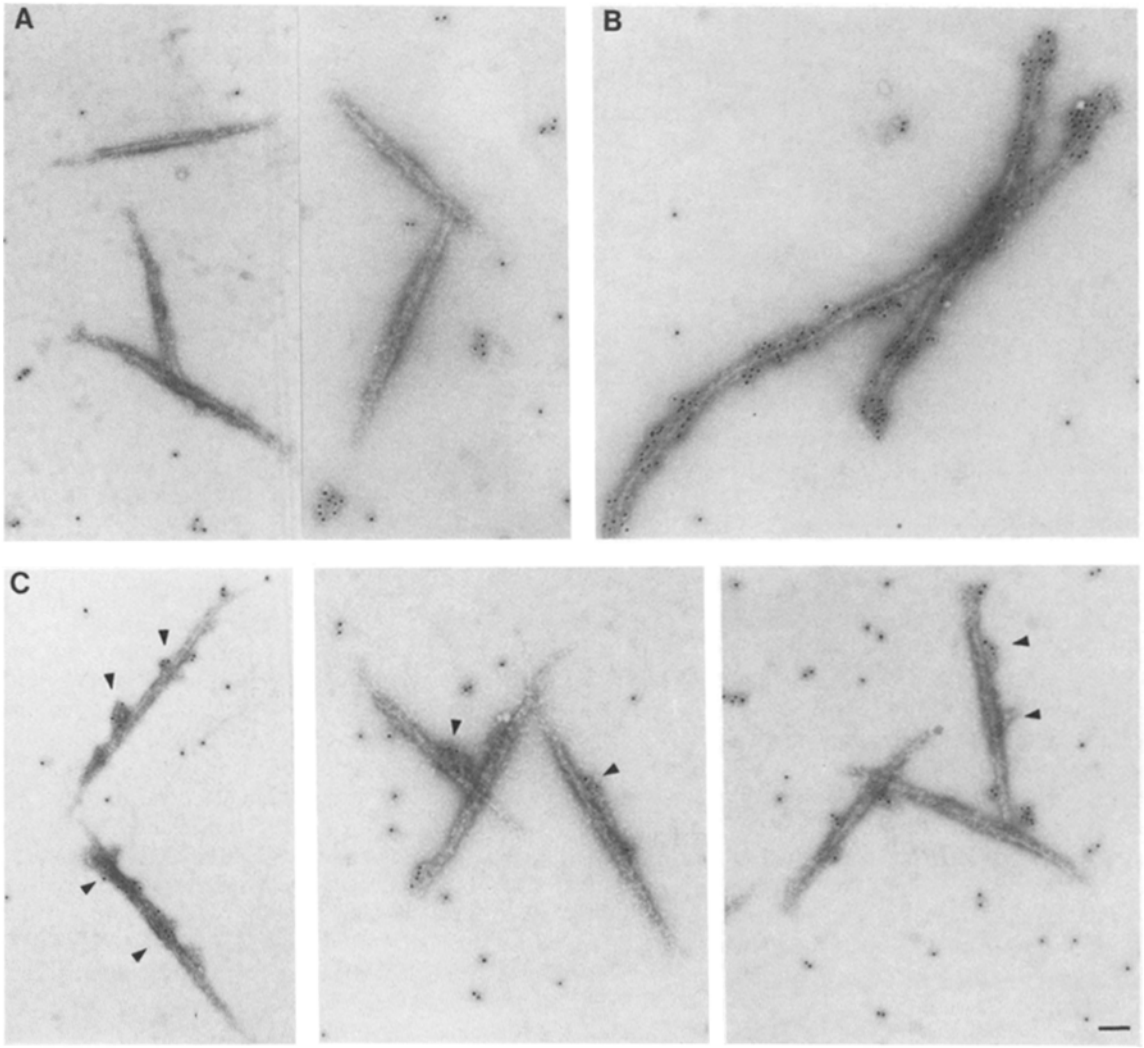


Figure 6. Exchange of skeletal myosin into smooth muscle myosin filaments. Controls showing that (*A*) smooth muscle myosin filaments are not labeled with gold after incubation with anti-pectoralis myosin IgG and gold-conjugated goat anti-rabbit IgG, but (*B*) skeletal myosin filaments are heavily labeled with gold along the filament length. (*C*) The critical concentration of skeletal muscle myosin was incubated with 30–300 μg/ml turkey gizzard myosin filaments overnight. Skeletal muscle myosin was incorporated along the length of the smooth muscle myosin filament as detected by the binding of the gold conjugated secondary antibody. Solvent: 5 mM citrate/16 mM Tris, 0.1 M KCl. Bar, 0.1 μm.

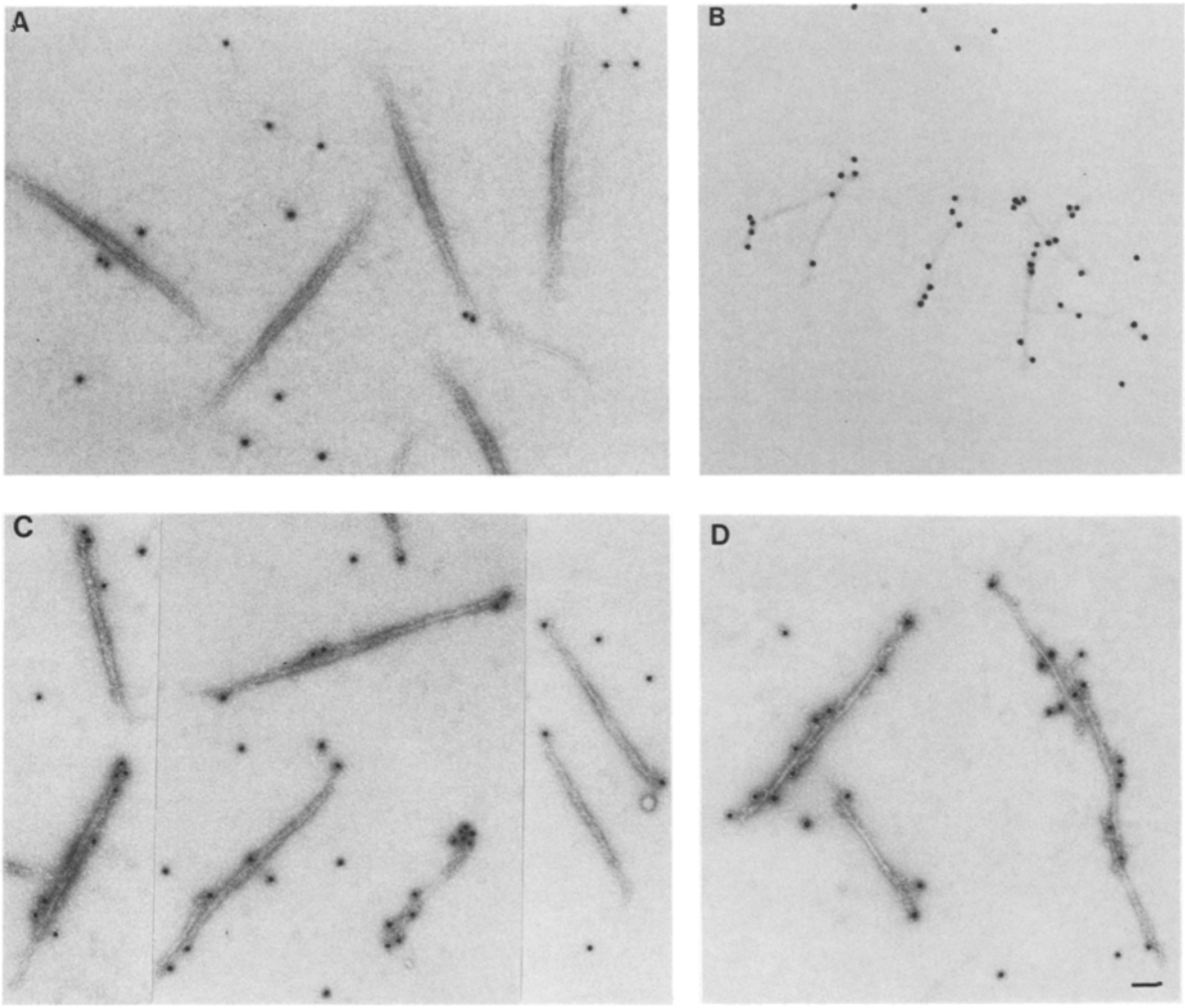


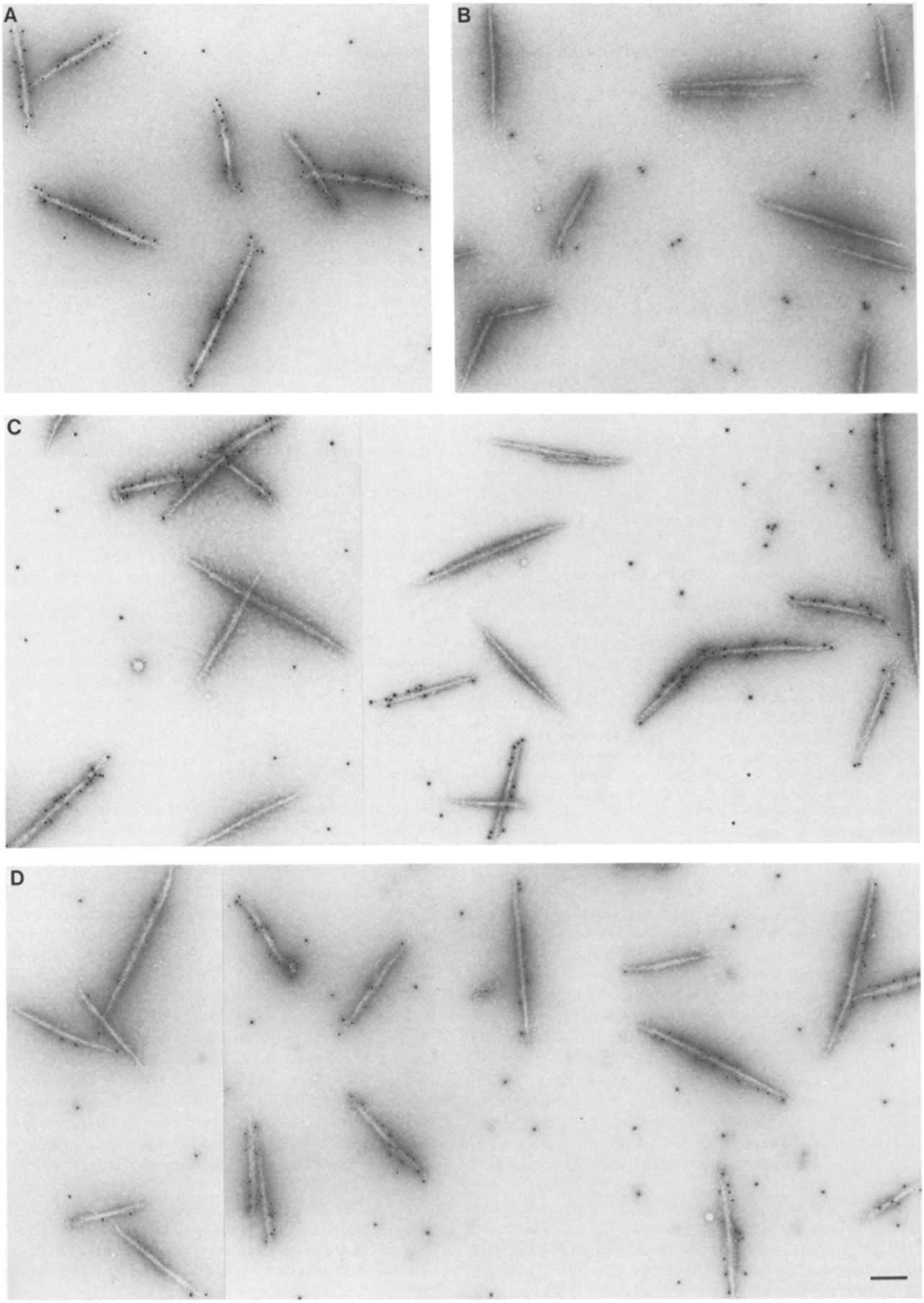
Figure 7. Exchange of myosin molecules between biotinylated and unmodified filaments. Controls showing that (*A*) unmodified turkey gizzard myosin filaments do not bind streptavidin-gold, but (*B*) filaments formed from biotinylated myosin are heavily labeled and considerably smaller (see Materials and Methods for details of the modification reaction). (*C*) Equal concentrations of the two populations of filaments (50 μg/ml total) were incubated for 2 min or (*D*) 60 min. Note the incorporation of gold into all filaments even at early times; by 60 min the filaments have a morphology intermediate between the unmodified and modified filaments. Solvent: 5 mM citrate/16 mM Tris, 0.1 M KCl. Bar, 0.1 μm.

not labeled with gold, whereas skeletal myosin filaments, which react with the primary antibody, are heavily labeled with gold along the filament length (Fig. 6 *B*). When the critical concentration of monomer in equilibrium with the skeletal muscle myosin filaments was incubated overnight with the smooth muscle myosin filaments, gold particles were bound along the length of the smooth muscle myosin filament, indicating that skeletal muscle myosin had been incorporated (Fig. 6 *C*).

Another approach was to follow the exchange of biotinylated smooth muscle myosin into unmodified smooth muscle myosin filaments. Biotinylated myosin was detected by the binding of streptavidin-gold. Fig. 7, *A* and *B*, shows that the control smooth muscle myosin filaments do not bind gold, while the filaments formed from biotinylated myosin, although considerably smaller, are heavily labeled with gold. After a 2-min incubation of equal amounts of control and biotinylated filaments, gold was detected on most filaments (Fig. 7 *C*). After a 60-min incubation, the filaments no longer resembled those formed by the control or the biotinylated myosin, in itself evidence of exchange. Moreover, by this time, all filaments in the field contain biotinylated myosin, as indicated by the bound gold particles (Fig. 7 *D*). In this experiment, the total protein concentration was low, 50 μg/ml, and exchange would be expected to be quite extensive because the critical concentration is being approached.

Exchange of molecules between unmodified and biotinylated filaments was further investigated at higher pro-

Figure 8. Exchange of biotinylated smooth muscle myosin into unmodified filaments at higher protein concentrations. Controls showing (*A*) heavy labeling of biotinylated filaments with streptavidin gold, (*B*) no labeling of unmodified filaments with gold, and (*C*) a 30-s incubation of equal amounts of unmodified and biotinylated filaments. (*D*) Equal concentrations of the two populations of filaments (1.3 mg/ml total) were incubated for 24 h. With time, all filaments contain biotinylated myosin as detected by the binding of streptavidin gold. Solvent: 10 mM imidazole, pH 7, 0.1 M KCl, 5 mM $MgCl_2$. Bar, 0.2 μm.


A
B
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D

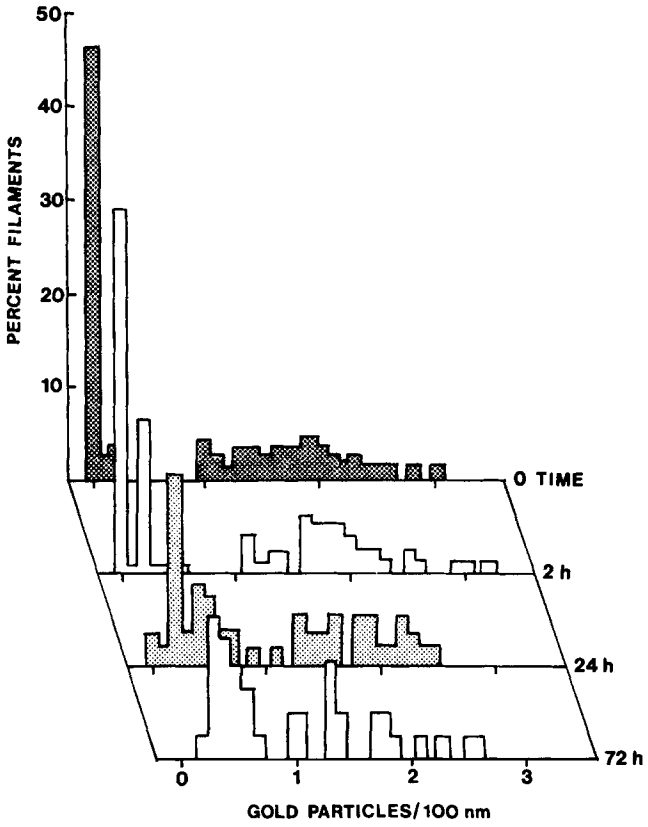

Figure 9. Incorporation of biotinylated myosin into unmodified filaments as a function of time. The results of the exchange experiment shown in Fig. 8 are presented in this histogram. The percentage of filaments containing the indicated density of gold particles is plotted for different times of incubation of the biotinylated and unmodified filaments. The number of gold particles are expressed per unit length because filament lengths are variable. Initially, two populations of filaments can be identified. With time, the number of unlabeled filaments decreases and all filaments show some degree of labeling.

tein concentrations to determine how the extent and time course of exchange are altered as the ratio of filament/monomer increases. In this case, a water-soluble, long-chain analogue of NHS-biotin was used for the modification reaction, and the morphology of the biotinylated filaments (Fig. 8 *A*) did not noticeably differ from that of the control filaments (Fig. 8 *B*). When equal concentrations of the two types of filaments were mixed (1.3 mg/ml total protein concentration) and incubated for <1 min, two populations of filaments could be clearly identified, indicating that exchange had not yet occurred to a measurable extent (Fig. 8 *C*). After a 24-h incubation, >95% of the filaments (n = 55) contained gold particles (Fig. 8 *D*), and by 72 h, all filaments contained biotinylated myosin. These results are graphically presented in the histogram shown in Fig. 9. There appears to be somewhat of a preference for exchange at filament ends, although with increasing time of incubation, the number of filaments that contained gold particles exclusively at the ends decreased from 30% at 24 h to 15% at 72 h. Overall, it appears that exchange is slower at higher protein concentrations, although variations in filament length and solvent conditions may be additional factors that affect the rate and extent of this process.

Discussion

When MgATP was added to copolymers of phosphorylated and dephosphorylated myosin, either as minifilaments or larger synthetic filaments, essentially all of the dephosphorylated myosin disassembled to the folded conformation. The lack of stabilization by neighboring phosphorylated molecules was unexpected, because intermolecular interactions might have been thought to contribute to the ability of the filament to remain assembled. The exchange of molecules between filaments described here provides a possible mechanism by which dissociation occurs. Phosphorylated myosin could leave and reenter the polymer in the presence of MgATP, but dephosphorylated myosin, once dissociated from the filament, would assume a stable folded conformation and be unable to reassemble (Cross et al., 1986). If molecules exchange freely in and out of the filament, neighboring molecules would not be expected to exert a stabilizing influence, and copolymers would have the same properties as mixtures of homopolymers. Another possibility is that the copolymer completely disassembles upon MgATP addition and is followed by reassembly of only the phosphorylated molecules. This mechanism, which cannot be excluded at present, could be distinguished from one in which there is a constant exchange of molecules by monitoring the kinetics of dissociation on a rapid time scale.

Evidence in favor of exchange of molecules was obtained in the absence of MgATP under conditions where filaments are assembled. When rod minifilaments were incubated with myosin minifilaments, copolymers were observed within 15 min of mixing the homopolymers. Given that the critical concentration of monomer in the minifilament buffer system is low (<25 μg/ml) compared with the filament concentration (1 mg/ml), the exchange of molecules between polymer and monomer pool appears to be quite extensive. It should be noted, however, that results obtained in one buffer system do not necessarily apply under all solvent conditions. Subunit exchange in F-actin, for example, was found to depend markedly on ionic conditions, and was very limited at approximately physiological ionic strength (Pardee et al., 1982). Another aspect to consider is that the small size of the minifilaments limits exchange to the ends of the filament, and leaves unanswered questions concerning the extent of exchange in larger filaments. Josephs and Harrington (1966) previously showed by sedimentation analysis that synthetic filaments are in rapid equilibrium with a monomer pool of skeletal muscle myosin. A similar monomer–polymer equilibrium was shown to exist for smooth muscle myosin (Megerman and Lowey, 1981), except that the polymerization behavior was difficult to analyze by a simple two-species system. The increased complexity was probably due to the fact that a mixture of phosphorylated and dephosphorylated myosin was present, and it was not appreciated at the time that these two species would have very different critical concentrations. These experiments established that exchange occurs in both skeletal and smooth muscle myosin filaments, but they could not distinguish between diffusional exchange restricted to filament ends, and an exchange occurring along the length of the filament.

In an attempt to assess the location and extent of exchange in larger filaments, gold-labeling techniques were used in conjunction with electron microscopy. By this approach, it was shown that molecules could be incorporated along the entire length of the filament, although at early times there appeared to be a preference for exchange at filament ends. The time course of exchange depended both on the protein concentration and on the solvent composition; thus, the rate of exchange that might occur in vivo cannot be realistically estimated. The major point established here is that subunit exchange does occur in myosin filaments, and is a process which can explain the disassembly by nucleotide.

At least three mechanisms can be considered to account for exchange along the length of a filament (see review by Fulton, 1985). Diffusional exchange is usually associated with random fluctuations at the ends of filaments, but the myosin filaments could break along their length and thereby create new ends for exchange before reannealing. Alternatively, molecules could exchange by diffusion along the sides of the filament. Assuming the simple packing of antiparallel dimers into a "side-polar" filament discussed in the accompanying paper, a dynamic equilibrium along the length of the filament is not too improbable. Assembly and disassembly at the ends of the filament, as postulated in a treadmilling mechanism, appears more unlikely in the case of myosin, in that nucleotide is not necessary for assembly, and no evidence exists to suggest that the ends of the filament have different critical concentrations. Clearly, a detailed kinetic analysis will be necessary to understand the assembly and exchange reactions in smooth muscle myosin.

Evidence for myosin exchange beyond the monomer–polymer equilibrium established by hydrodynamic analysis (Josephs and Harrington, 1966) has already been presented for skeletal muscle myosin. Using fluorescence energy transfer or radioisotope-labeled myosin, Saad et al. (1986) showed extensive exchange in this system. They suggested that exchange may be a mechanism by which turnover occurs in a highly ordered structure, the myofibril. Individual proteins in the myofibril have characteristic turnover rates (Zak et al., 1977), indicating that a mass destruction and reassembly of whole sarcomeres probably do not occur. Moreover, throughout development there must be a mechanism by which the stage-specific myosin isoforms are sequentially added to the myofibril and then replaced by the isoform characteristic of the next developmental stage. Myosin turnover in vivo was recently investigated by using thyroid hormone to control the level of synthesis of the α and β isoforms of cardiac heavy chain. Analysis of the spatial and temporal reincorporation of α myosin heavy chain into thick filaments in cardiac myocytes showed that newly synthesized myosin preferentially added onto filament ends, but was also incorporated along the filament length (Wenderoth and Eisenberg, in press).

The observation of exchange of molecules between monomer pool and polymer has been studied much more extensively with cytoskeletal proteins other than myosin. The exchangeability of α-actinin (McKenna et al., 1985), actin (Kreis et al., 1982), and tubulin (Salmon et al., 1984) has been examined in living cells by microinjection of fluorescently labeled proteins, followed by photobleaching. The rate of recovery of fluorescence is considered to be a measure of the rate at which proteins assemble and disassemble from cellular structures. With α-actinin and actin, it appears that the degree of exchangeability of the protein may depend on its exact molecular environment. α-Actinin, for example, appears to be more stably associated with the Z-band in the myofibril than with adhesion plaques and stress fibers in fibroblasts, but even in the same cell different rates of fluorescence recovery are measured (McKenna et al., 1985).

The properties of smooth muscle myosin are, in many respects, similar to those of nonmuscle myosins; exchange of molecules may prove to be another common property. The cytoskeleton must be rearranged during cell locomotion and changes in cell shape, and turnover in the myosin filaments as well as in actin polymers and microtubules could be envisioned during these cellular events. The microinjection and fluorescence techniques described above will be needed to determine if myosin in smooth and nonmuscle cells is as readily exchangeable as the in vitro experiments suggest.

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