

# Association of Microinjected Myosin and Its Subfragments with Myofibrils in Living Muscle Cells

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**Abstract.** Purified skeletal muscle myosin was labeled with iodoacetamidofluorescein and microinjected into cultured chick myotubes. The fluorescent myosin analogue became incorporated within 10–15 min after injection, into either periodic (mean periodicity =  $2.23 \pm 0.02 \mu\text{m}$ ) bands or apparently continuous fibrillar structures. Comparison of rhodamine-labeled alpha-actinin with coinjected fluorescein-labeled myosin suggested that myosin fluorescence was localized at the A-bands of myofibrils. In addition, close examination of the fluorescent myosin bands indicated that they were composed of two fluorescent bars separated by a nonfluorescent line that corresponded to the H-zone. Once incorporated, the myosin underwent a relatively slow exchange along myofibrils as indicated by fluores-

cence recovery after photobleaching. Glycerinated myofibrils were able to bind fluorescent myosin in a similar pattern in the presence or absence of MgATP, indicating that actin–myosin interactions had little effect on this process. Fluorescent heavy meromyosin did not incorporate into myofibrillar structures after injection. Light meromyosin, however, associated with A-bands as did whole myosin. These results suggest that microinjected myosin, even with its relatively low solubility under the cytoplasmic ionic condition, is capable of association with physiological structures in living muscle cells. Additionally, the light meromyosin portion of the molecule appears to be mainly responsible for the incorporation.

**M**YOSIN, the main component of the thick filament in myofibrils, is one of the best-characterized proteins (Craig, 1986; Harrington and Rodgers, 1984; Huxley, 1963). The molecule consists of two globular heads, which are responsible for its enzymatic properties and interaction with actin, and a rod-like tail portion (hereafter referred to as rod), which can be further cleaved into light meromyosin (LMM)<sup>1</sup> and an S2 region.

A well-known characteristic of skeletal muscle myosin is its relatively low solubility, due to the self-assembly of synthetic filaments, under physiological conditions. It is commonly recognized that the rod portion of the molecule plays an important role in this self-assembly process. For example, both LMM (Strzelecka-Golaszewska et al., 1985) and rod (Harrington and Himmelfarb, 1972) can form synthetic thick filaments closely resembling filaments of whole myosin. Recent studies further indicate that a crucial site for self-assembly is located in the LMM portion, close to the carboxyl terminal (Nyitray et al., 1983). The role of the heads in filament formation is not nearly as clear. On the one hand, heavy meromyosin (HMM), a myosin subfragment that contains the heads and S2, is unable to self-assemble, implying

that the head portion has little effect on filament formation. On the other hand, experiments with synthetic thick filaments suggest that the MgATPase sites on the heads may modulate the assembly process (Pinset-Harstrom, 1985).

Questions arise when one tries to correlate the biochemical properties of myosin in vitro with cellular structures and functions. For example, we do not know whether myosin molecules can diffuse in the cell and become associated with appropriate structures. In fact, some authors have postulated that myosin and other contractile proteins may become associated with cellular structures as soon as they are synthesized (Isaacs and Fulton, 1987). It is also important to know how thick filaments are assembled in living cells, which portion of the molecule is responsible for the assembly, and whether there is a dynamic equilibrium of the molecules between assembled and unassembled states.

One way to address these questions is to microinject fluorescently labeled myosin or its subfragments into living cells and examine the behavior of these fluorescent analogues in relation to physiological structures. In this study, we demonstrate that fluorescently labeled skeletal muscle myosin, even with its low solubility under physiological ionic conditions, can nevertheless disperse and incorporate into myofibrils after microinjection into living chick myotubes. A similar pattern of incorporation was observed with fluorescently labeled LMM. Microinjected HMM, however, was

1. *Abbreviations used in this paper:* FRAP, fluorescence recovery after photobleaching; HMM, heavy meromyosin; IAF, iodoacetamidofluorescein; IATR, tetramethylrhodamine iodoacetamide; LMM, light meromyosin.

unable to associate with any myofibrillar structures. We have also examined the exchange of myosin along myofibrils at steady-state using fluorescence recovery after photobleaching (FRAP). Our data suggest that once myosin is associated with thick filaments, exchange occurs at a relatively slow rate.

## Materials and Methods

### Preparation of Fluorescent Analogues

Skeletal muscle myosin was isolated from rabbit back and hind leg muscles according to the protocol of Pollard (1982) with modifications suggested by Margossian and Lowey (1982), using low salt precipitations and ammonium sulfate cuts to separate the myosin from other proteins. The purified myosin was stored in 50% ammonium sulfate at a concentration of 20–40 mg/ml at 4°C. HMM was prepared by enzymatic cleavage of myosin with alpha-chymotrypsin (Cooper Biomedical, Inc., Malvern, PA; Margossian and Lowey, 1982).

Fluorescent labeling of myosin and HMM was performed by dissolving iodoacetamidofluorescein (IAF; Molecular Probes, Inc., Eugene, OR) in 0.5 M KCl, 0.05 M Hepes, pH 7.5–8.0, and adding the solution to the protein in the same buffer at a dye to protein molar ratio of 30–50:1. After brief mixing, the solution was incubated 1–2 h at 0°C in the dark, clarified, and applied to a column of Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) to remove unbound dye molecules. Fluorescently labeled myosin was further dialyzed against a low salt buffer, and centrifuged to pellet the fluorescent myosin. This step not only concentrates the sample, but also selects for the fraction of fluorescent protein that maintains the ability to self-assemble. The pellet was resuspended in a small amount of 2 M KCl, and dialyzed against an injection buffer containing 0.45 M KCl and 2.0 mM Pipes, pH 6.95. The IAF-labeled myosin had a final dye to protein molar ratio of 1–6:1, based on a molar extinction coefficient of 60,000 at 495 nm, pH 8.0, for bound fluorescein. Most experiments were performed using conjugates with a labeling ratio of 1.5–3.0. The labeled myosin was stored for up to 2 d on ice and was clarified before injection.

Fluorescent LMM and rod were prepared by enzymatically digesting myosin that had been labeled at a ratio >2.0. LMM was prepared by cleavage of myosin with alpha-chymotrypsin (Margossian and Lowey, 1982). Rod was prepared by a similar procedure in a low salt, EDTA-containing buffer (Margossian and Lowey, 1982). Both LMM and rod were purified by ethanol precipitation followed by assembly–disassembly in low and high salt buffers. The dye to protein molar ratio for rod and LMM was 0.8–2.1:1 and 0.3–0.7:1 respectively. Labeled subfragments were handled similarly to the procedure described for whole myosin.

Smooth muscle alpha-actinin was isolated from frozen turkey gizzards and labeled with tetramethylrhodamine iodoacetamide (IATR; Molecular Probes, Inc.) as described previously (Meigs and Wang, 1986).

### Biochemical Assays of Fluorescently Labeled Myosin

SDS-PAGE was performed according to Laemmli (1970). A 10% gel was used for myosin and subfragments, and a 15% gel for peptides generated by limited tryptic digestion of S1.

S1 was prepared from labeled myosin with alpha-chymotrypsin according to Margossian and Lowey (1982), except that S1 was purified from the soluble fraction by an ammonium sulfate cut between 47 and 58% saturation. Limited tryptic digestion of S1 was performed by adding TPCK-treated trypsin (Cooper Biomedical, Inc.) at a trypsin/S1 weight ratio of 1:100 in a buffer of 50 mM KCl, 50 mM Tris-HCl, pH 8.0. After incubation for 2, 5, and 10 min at room temperature, samples were removed, mixed with the sample buffer for electrophoresis, and heated in a boiling water bath for 2 min.

ATPase activities were assayed following the protocol of Pollard (1982). Self-assembly of myosin was studied with both turbidity measurements and electron microscopy. Myosin at a concentration of 9–11 mg/ml was diluted to obtain a final concentration of 0.1–0.8 mg/ml in a buffer of 150 mM KCl, 2 mM MgCl<sub>2</sub>, 0.2 mM dithiothreitol, and 20 mM Pipes, pH 7.0, for turbidity measurements at 320 nm; and a final concentration of 40 µg/ml in a buffer of 50 mM KCl, 2 mM MgCl<sub>2</sub>, 5 mM Pipes, pH 7.0, for negative staining.

## Cell Culture, Microinjection, and Fluorescence Microscopy

Muscle cell cultures were prepared and maintained according to McKenna et al. (1986). Myotubes were injected 5–8 d after plating as described previously (Wang, 1984; McKenna et al., 1986), at several points along their length. This method of microinjection delivers as much as 5–10% of the cell volume (Wang et al., 1982). The estimation of injection volume was difficult for myotubes, which often have a branched, extended morphology. However, the actual volume injected was significantly less than the normal limit due to the sensitivity of living cells to the high salt in the carrier solution. Myosin and subfragments were microinjected at a concentration of 6–8 mg/ml.

After microinjection, myotubes were incubated 2–4 h in a CO<sub>2</sub> incubator, and then observed as described previously (McKenna et al., 1986). When rhodamine and fluorescein conjugates were used simultaneously, an additional barrier filter (cutoff wavelength 550 nm) was used with fluorescein filters to avoid crossover of rhodamine fluorescence. The low level of excitation light did not cause detectable changes in cellular morphology, as observed with phase or fluorescence microscopy.

Glycerination of muscle was performed according to the procedure of Sanger et al. (1984). The plates were washed with a rinsing buffer of 100 mM KCl, 0.5 mM EGTA, 5 mM MgCl<sub>2</sub>, and 5.0 mM Pipes, pH 7.0; with or without 3 mM ATP. The rinsing buffer was removed and fluorescent proteins, also in buffers with or without ATP, were then added to the glycerinated cells and incubated for 30 min. The carrier buffer for fluorescent myosin and its subfragments contained 0.45 M KCl to ensure that the conjugate was in the soluble form during the incubation. The plates were rinsed with rinsing buffer to remove excess fluorescent proteins before microscopic observations.

### Image Processing and Analysis

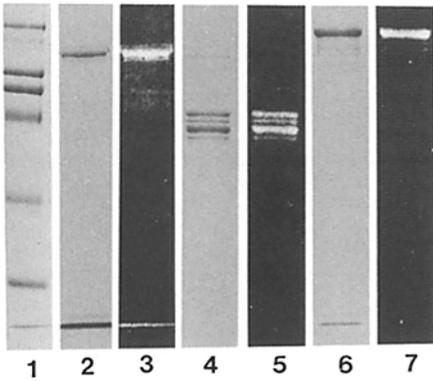
Hardware used for detection and storage of fluorescent images was identical to that described previously (McKenna et al., 1986). Distances were measured with a graphics tablet (GTCO, Rockville, MD) as described in McKenna et al. (1986). Cells were photobleached with the 488-nm line of a 2-W argon ion laser (Lexel Corp., Palo Alto, CA), using equipment identical to that reported in McKenna et al. (1985). The laser pulse, which had a power of 25 mW and a duration of 50 ms, did not disrupt the integrity of myofibrils, as determined by rhodamine–phalloidin staining (McKenna et al., 1985). To correct for photobleaching during image recording, images of a recovery series were normalized to obtain a similar average intensity outside the bleached area. Statistical analyses are reported as mean ± SE or SD, as noted.

## Results

### Characterization of Fluorescently Labeled Myosin and Its Subfragments

The purity of the labeled myosin and its subfragments was determined by SDS-PAGE (Fig. 1). The pattern of bands was similar to that documented by Weeds and Pope (1977). Unbound fluorescein, which moves slightly ahead of the tracking dye, was undetectable.

The fluorescent dye used, 5-IAF, reacts predominantly with cysteine sulfhydryl groups. Results from several types of experiments were used to identify the primary sites of reaction. First, the most reactive sites appeared to locate at S1 heads, which were labeled stoichiometrically when the whole myosin was labeled with more than two probes. Second, when S1, labeled at a stoichiometry of 1:1, was further digested with trypsin, most probes were located in one single peptide with a molecular weight of 20,000 (Fig. 2). This peptide was previously shown to contain two reactive sulfhydryl groups, SH-1 and SH-2 (Balint et al., 1978). Third, it is known that when SH-1 is modified, the Ca-ATPase becomes activated whereas the K-EDTA-ATPase is inhibited. Modification of both SH-1 and SH-2 causes inhibition of both

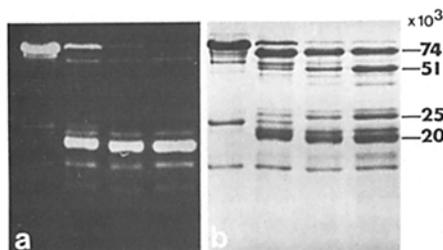


**Figure 1.** SDS-PAGE of fluorescently labeled proteins. Lane 1, standard, consisting of myosin heavy chain (205,000), beta-galactosidase (116,000), phosphorylase b (97,400), BSA (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), and unresolved myosin light chains moving at the front; lane 2, HMM stained by Coomassie Blue; lane 3, fluorescence of HMM; lane 4, LMM stained by Coomassie Blue; lane 5, fluorescence of LMM; lane 6, myosin stained by Coomassie Blue; lane 7, fluorescence of myosin.

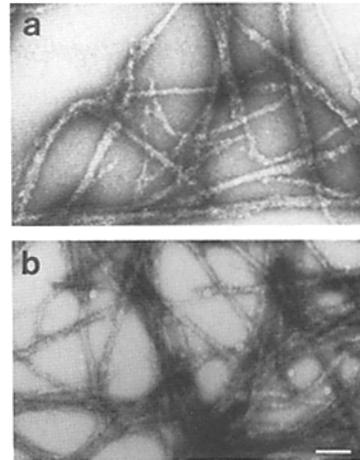
K-EDTA- and Ca-ATPase activities (Reisler, 1982). Our labeled myosin showed a fivefold activation of the Ca-ATPase (from 0.42  $\mu\text{mol}/\text{min mg}$  to 2.3  $\mu\text{mol}/\text{min mg}$  at 30°C) and a 20-fold inhibition of the K-EDTA-ATPase (from 3.7  $\mu\text{mol}/\text{min mg}$  to 0.19  $\mu\text{mol}/\text{min mg}$  at 30°C), indicating that SH-1 was the primary site of labeling.

The actin-activated ATPase activity decreased from 1.4  $\mu\text{mol}/\text{min mg}$  for unlabeled myosin to 0.11  $\mu\text{mol}/\text{min mg}$  for IAF-labeled myosin, when measured in the presence of 0.8 mg/ml actin at 25°C. A similar decrease (90–95%) was observed at other actin concentrations. Consistent results were also obtained in previous studies of sulfhydryl-modified myosins (Mulhern and Eisenberg, 1978; Mulhern et al., 1975; Takashi et al., 1976).

The self-assembly of labeled myosin was examined both qualitatively with electron microscopy and quantitatively with turbidity measurements at 320 nm. Synthetic filaments of labeled myosin were indistinguishable from those of unlabeled myosin under the electron microscope (Fig. 3). In addition, both labeled and unlabeled myosins had an undetectable critical concentration and showed a similar increase of turbidity as a function of concentration (Fig. 4), in a buffer mimicking physiological conditions. When LMMs were



**Figure 2.** SDS-PAGE of fluorescently labeled S1 that has been further digested by trypsin. Fluorescence (a) and corresponding Coomassie Blue-stained image (b) show S1 before, and 2, 5, and 10 min after digestion (from left to right). Molecular weights are indicated.



**Figure 3.** Negative-stained electron microscopic images of fluorescently labeled (a) and unlabeled (b) synthetic myosin filaments. Bar, 0.1  $\mu\text{m}$ .

tested under the same condition, labeled LMM showed both a lower apparent critical concentration and a higher turbidity as compared to unlabeled controls (Fig. 4).

### Microinjection of Fluorescently Labeled Myosin into Embryonic Chick Myotubes

Immediately after injection of the fluorescently labeled myosin, small fluorescent aggregates often formed at the injection site. However, most fluorescent molecules dispersed from the site of injection and became localized in fiber structures within 20 min after injection. Between 0.5 and 3 h, diffuse fluorescence decreased and myosin incorporation reached an apparently steady state. Only cells that appeared normal with phase-contrast microscopy were used for observations. Like uninjected cells, injected cells underwent occasional spontaneous contractions. However, the irregularity of the contraction made it difficult to compare the frequency quantitatively.

In immature myotubes, 3–5 d in culture, myosin was localized primarily in periodic bands (Fig. 5 a), but sometimes the fluorescence appeared continuous (Fig. 5 a, arrow) along all or parts of the fiber structures. In more mature myotubes, cultured for 6–8 d, myosin was localized in wide bands (1.59  $\mu\text{m}$ , SE = 0.03; Fig. 5 b) with a periodicity of 2.23  $\mu\text{m}$  (SE = 0.02). Under favorable conditions, a dark line dividing the wide bands into doublets could be detected (Fig. 5 b; see also Fig. 12 a). The pattern of incorporation was unaffected by the stoichiometry of fluorescent labeling and the amount of microinjection.

IATR-labeled gizzard alpha-actinin, which localizes at the Z-line of myofibrils (Kreis and Birchmeir, 1980), was co-injected with IAF-labeled myosin into some myotubes. As shown in Fig. 5, b and c, myosin bands were located between alpha-actinin-containing Z-lines, suggesting that myosin was associated with the A-bands. Additionally, incorporated fluorescent myosin remained associated with the A-bands after glycerination in the presence of MgATP (not shown),

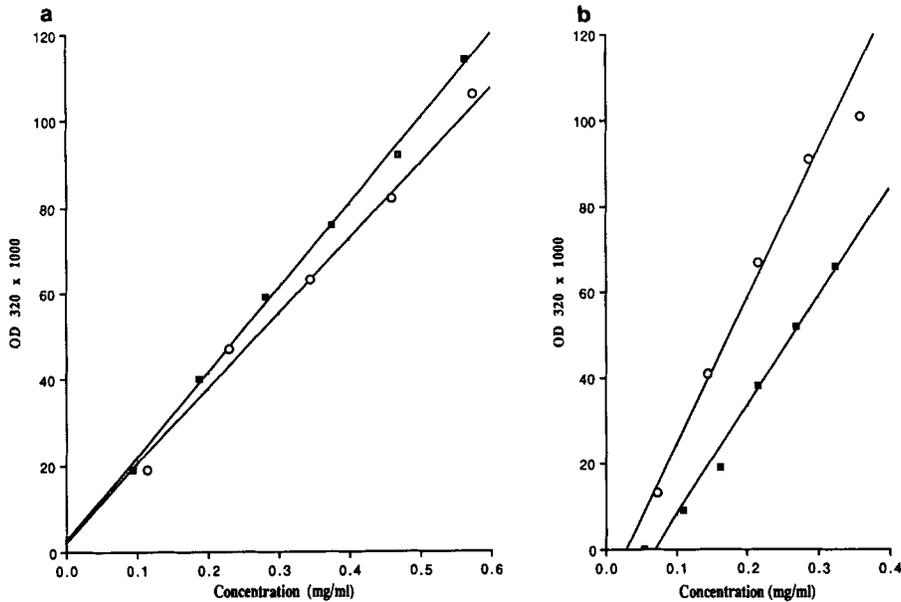


Figure 4. Self-assembly of labeled and unlabeled myosin (a) and LMM (b). Myosin or LMM was diluted to the specified concentrations and the optical density measured at 320 nm. Optical densities of unassembled myosin at corresponding concentrations were subtracted from each value. (■) Unlabeled; (○) IAF-labeled.

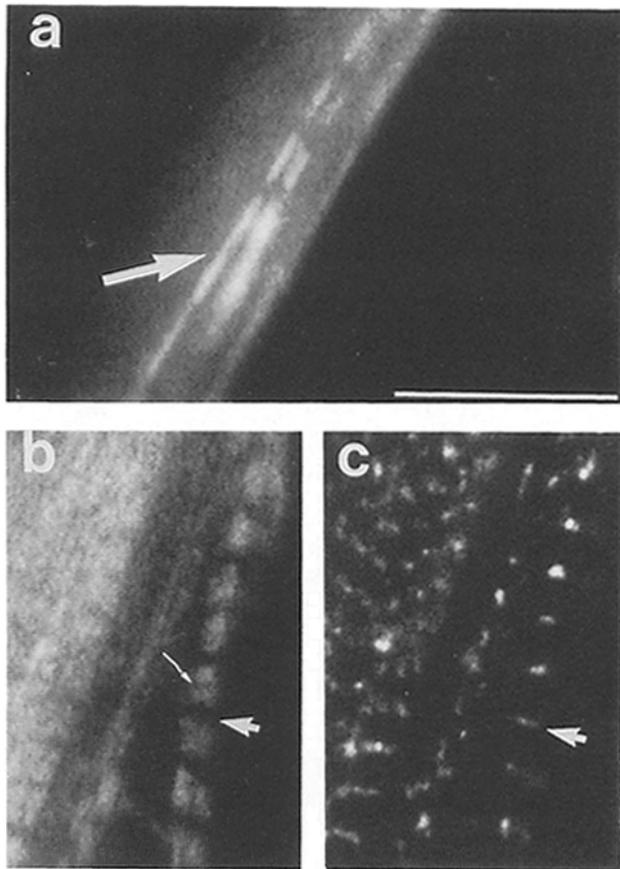


Figure 5. Localization of microinjected IAF-labeled myosin in chick skeletal myotubes. In a relatively immature myotube (4 d in culture; a), fluorescent myosin was observed in both periodic bands and continuous segments (arrow) along myofibrils. In a relatively mature myotube (7 d in culture; b and c), fluorescent myosin (b) was localized in periodic bands with a dark line in the middle (b, small arrow). Microinjection of IATR-labeled alpha-actinin into the same myotube revealed the location of Z-lines (c). Comparison of the distributions of the two proteins indicated that fluorescent myosin was localized in the A-bands of myofibrils. Large arrows, corresponding locations of the same Z-line. Bar, 10  $\mu$ m.

suggesting that the association of fluorescent myosin with myofibrils was not maintained by actin-myosin interactions.

#### FRAP of IAF-labeled Myosin in Myofibrils

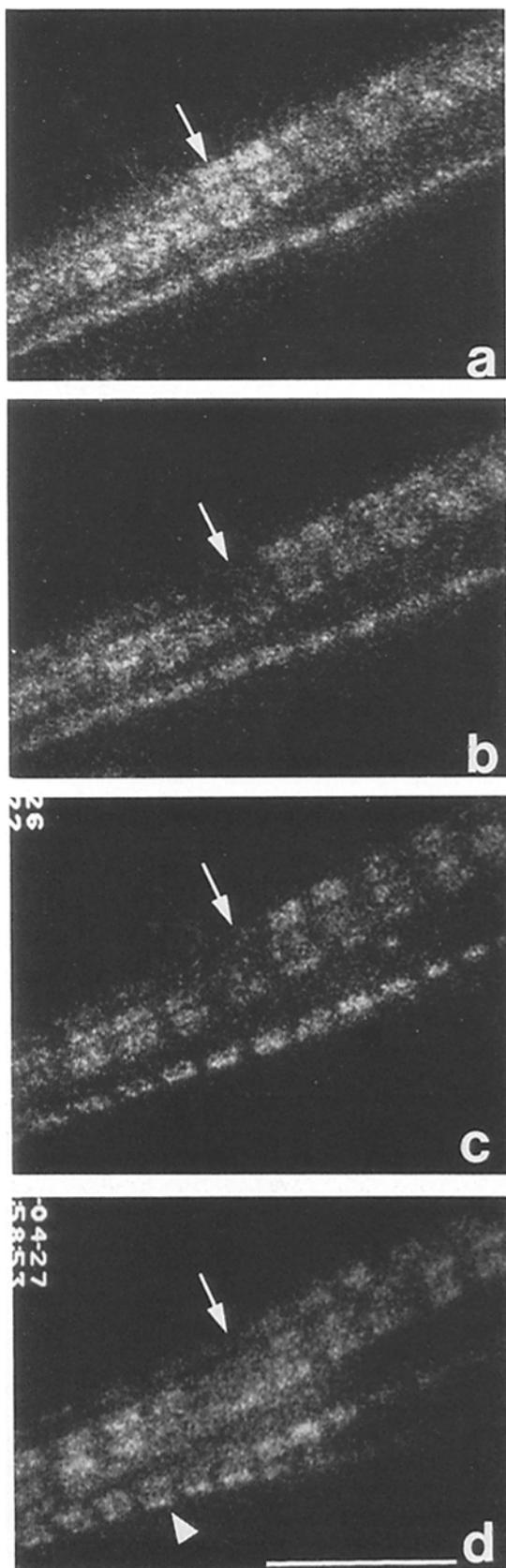
We were also interested in whether fluorescent myosin, once incorporated into the A-bands of myofibrils, could undergo exchange. Therefore, fluorescent sarcomeres were photobleached with a laser microbeam and the images of the cell recorded subsequently. As shown in Fig. 6, the bleached spot remained detectable as long as 18 h after photobleaching. Myofibrillogenesis appeared to continue during this observation period, as evidenced, for example, by an increase in the width of the lower myofibril (arrowhead) in Fig. 6 d. Similar increases in width have been observed previously with fluorescently labeled alpha-actinin (McKenna et al., 1986).

We have also estimated the approximate extent of fluorescence recovery. Since the fluorescein fluorophore is easily photobleached during routine observations, the integrated intensity in the bleached area was normalized against that in unbleached areas. Of the three experiments performed, the mean percentage recovery at 4 h was 22.3% (SD = 5.6). The recovery increased slowly over 18 h to 65–70%. These results suggest that once injected myosin was incorporated into myofibrils, the binding was relatively stable.

#### Association of IAF-Myosin with Glycerinated Myofibrils

When glycerinated cells were incubated with IAF-labeled myosin, a pattern identical to that in living cells was observed (Fig. 7 a). Comparison of myosin images to alpha-actinin images indicated that the bands of myosin were located between, but not associated with, Z-lines (Fig. 7 b). The localization of fluorescence at A-bands was further confirmed using glycerinated snake adult skeletal muscle, which has easily recognizable A-bands under phase optics. As shown in Fig. 8, the fluorescent bands colocalized with the phase-dense A-bands.

To examine the possible effect of ATP on the binding of labeled myosin, similar experiments were performed in the presence of 3 mM ATP and  $MgCl_2$ . As shown in Fig. 7, c



**Figure 6.** FRAP of IAF-labeled myosin localized in the A-bands of a myofibril. Fluorescence images are shown before (a), immediately after (b), 4 h after (c), and 18 h after (d) photobleaching. The

and d, the binding was not affected by the presence of MgATP, suggesting that actin–myosin interactions are not necessary for the association of myosin in glycerinated cells.

### **Localization of Myosin Subfragments in Living and Glycerinated Myotubes**

Fluorescently labeled myosin subfragments were prepared and microinjected into living myotubes to determine which portion of the myosin molecule is responsible for its incorporation into myofibrils. When IAF-labeled HMM was microinjected into living chick myotubes, it failed to associate with myofibrils or any other recognizable structures (Fig. 9 a), although well-developed sarcomeres were present in the corresponding alpha-actinin images (Fig. 9 b). This suggests that the HMM portion of myosin is insufficient for incorporation into fibrillar structures of living myotubes.

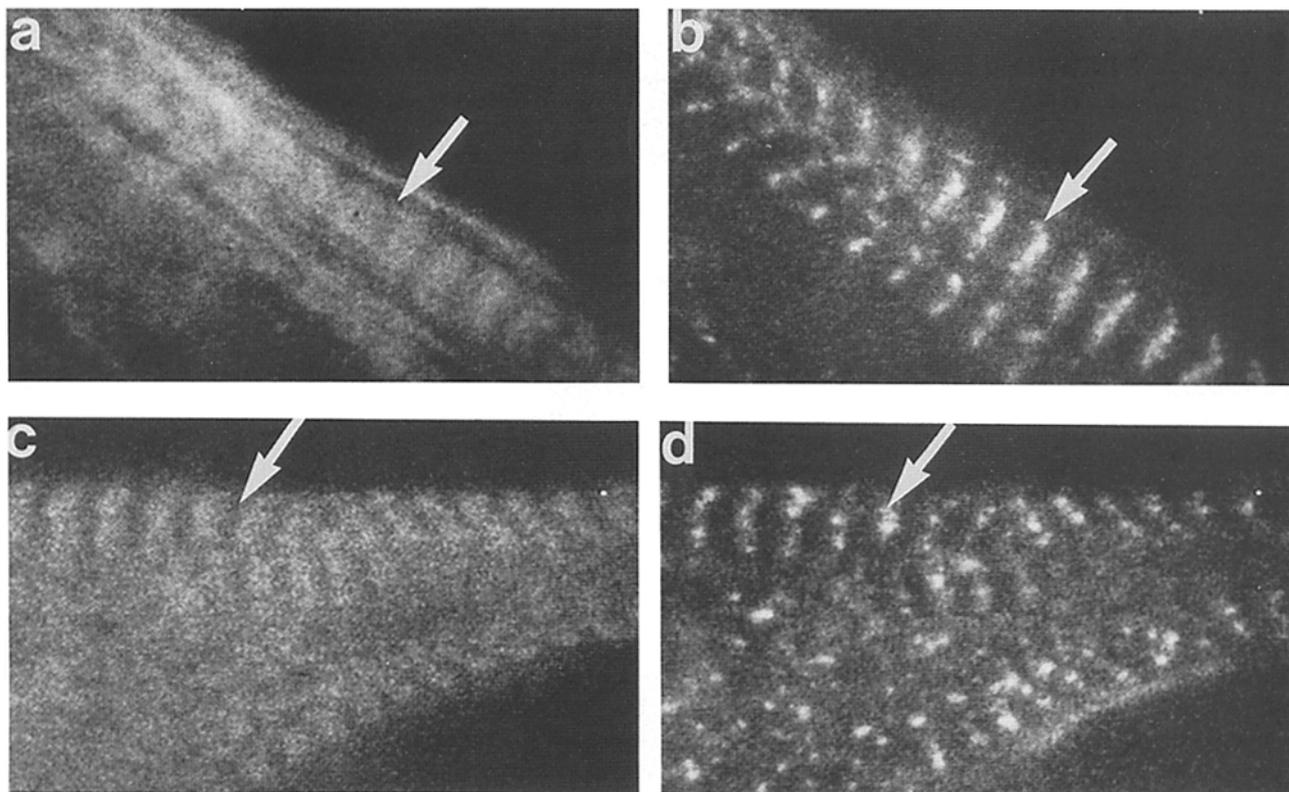
When fluorescent HMM was applied to glycerinated myotubes in the absence of ATP, bright fluorescent bands were detected in the region of the I-band (Fig. 10 a), as evidenced by the corresponding alpha-actinin image (Fig. 10 b). However, in the presence of MgATP, HMM showed only faint diffuse fluorescence (Fig. 10 c), although the corresponding alpha-actinin image indicated the presence of well-developed myofibrils (Fig. 10 d). These results suggest that the binding of HMM in the absence of ATP is probably due to the decoration of actin filaments in the I-band, and not to the association of the fragment with the thick filament.

When fluorescent LMM, prepared by proteolytic digestion of IAF-labeled myosin, was microinjected into myotubes, discrete bands similar to those seen with injected whole myosin were visible (Fig. 11 a). After glycerination of cells microinjected with fluorescent LMM and subsequent application of IATR-labeled alpha-actinin, it was clear that the labeled LMM was localized at the A-band (Fig. 11, b and c). Application of IAF-labeled LMM and IATR-labeled alpha-actinin to glycerinated myotubes yielded similar results (Fig. 12, a and b). Experiments conducted with IAF-labeled rod (not shown) resembled those attained with LMM and whole myosin.

### **Discussion**

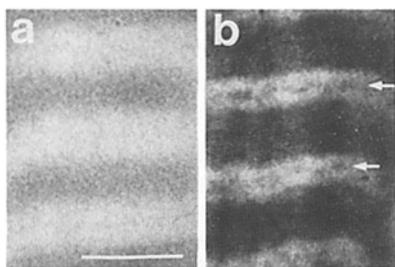
We have microinjected myosin molecules that had been labeled with fluorescein and cycled through assembly and disassembly. The labeled myosin polymerized normally as judged by electron microscopy and turbidity measurements. Based on various assays, it appears that the probes were located primarily at SH-1 of the S1 head and also in the rod region, whereas SH-2 did not appear to be affected. Although the labeling of SH-1 has caused a decrease in the

bleached area (arrows) remains detectable as a dark spot throughout the period of observation. Note that the lower myofibril (d, arrow-head) has increased in width during this period. Bar, 10  $\mu$ m.



**Figure 7.** Presence of ATP does not affect the A-band association of IAF-labeled myosin applied to glycerinated myotubes. Glycerinated myotubes incubated sequentially with IAF-labeled myosin (*a*) and IATR-labeled alpha-actinin (*b*) in the absence of ATP showed myosin fluorescence in doublets at the A-band. When 3 mM ATP was included in the incubation buffer, staining with fluorescent myosin (*c*) and alpha-actinin (*d*) resulted in the same pattern. *Arrows*, corresponding locations of the same Z-line for each pair. Bar, 10  $\mu$ m.

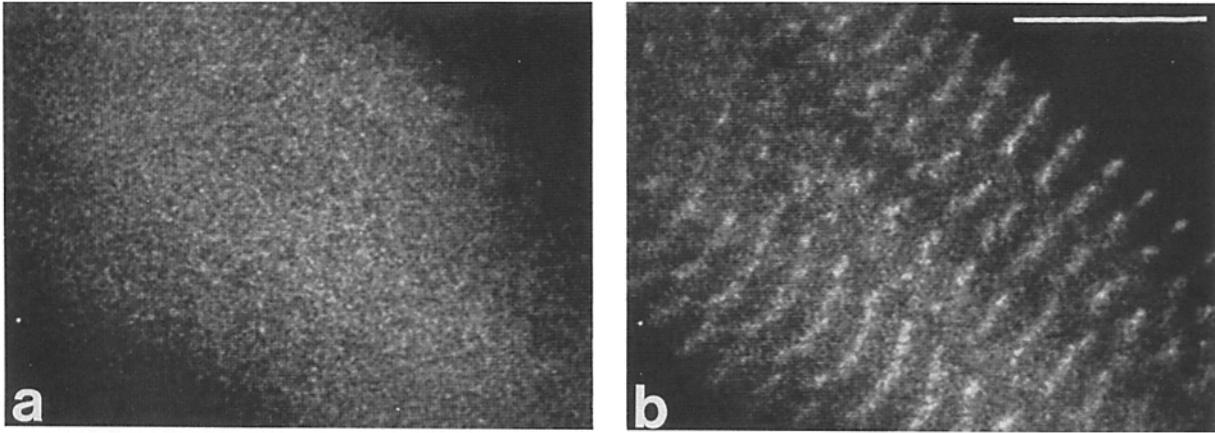
actin-activated MgATPase activity, at least some of the actin-binding properties were maintained. For example, the IAF-labeled HMM bound the I-bands of glycerinated myofibrils in a MgATP-sensitive fashion (Fig. 10). In addition, other investigators have observed the maintenance of myofibril contraction after similar modifications of myosin sulphydryl groups (Crowder and Cooke, 1984).



**Figure 8.** Incorporation of fluorescently labeled myosin into glycerinated snake myofibrils. The myofibrils were incubated with 12 mg/ml IAF-labeled myosin, rinsed extensively, and observed under fluorescence (*a*) and phase (*b*) optics. Phase-dark A-bands are labeled with the fluorescent myosin. *Arrows* in *b* indicate Z-lines. Bar, 5  $\mu$ m.

Our results demonstrate that, after microinjection, fluorescent myosin can incorporate into continuous or periodic fibrillar structures of immature muscle cells, and into periodic bands of myofibrils in mature myotubes. Therefore, even though fluorescent myosin is relatively insoluble under physiological ionic conditions, it is capable of diffusing away from the injection site in the cytoplasm. In addition, comparison of these results with immunofluorescence studies (Fallon and Nachmias, 1980; Kulikowski and Manasek, 1979) indicate that injected myosin closely mimics the localization of endogenous myosin. The size of fluorescent myosin bands in microinjected cells also corresponds to that reported by others for A-bands in avian muscle (Fischman, 1970). The dark line separating the fluorescent band into doublets may be due to the preferential location of the fluorescein probe in the head region of myosin molecules. However, since similar results were obtained with labeled LMM, it is likely that other factors, such as a possible inhibition of incorporation into the M-line region due to the presence of accessory proteins, may contribute to the appearance of the dark line. Recent studies on the incorporation of myosin into thick filaments *in vivo* also indicate a preferential association of nascent chains with the ends of thick filaments (Wenderoth and Eisenberg, 1987).

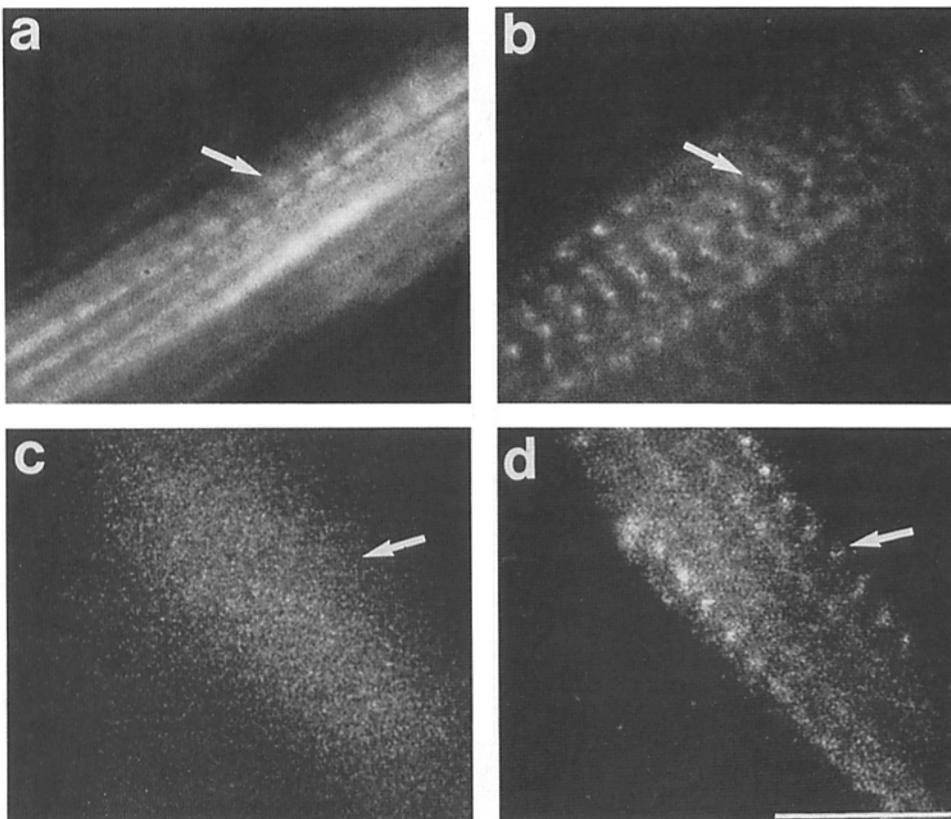
We observed a slow recovery of fluorescence after photobleaching fluorescent myosin that had incorporated into the



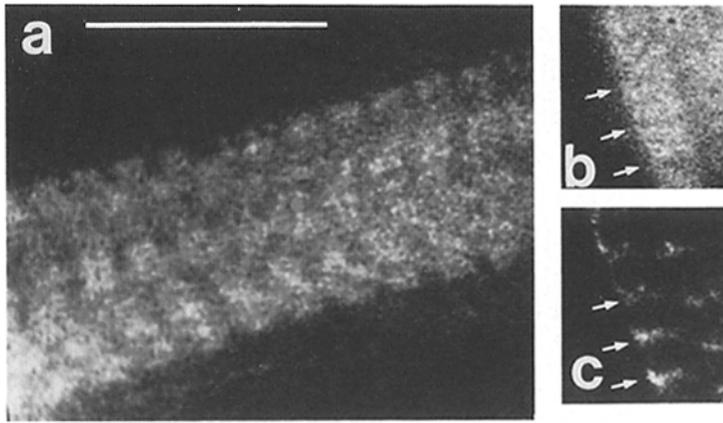
**Figure 9.** Microinjected IAF-labeled HMM does not incorporate into myofibrils of living chick myotubes. HMM was prepared by enzymatic cleavage of myosin followed by labeling with IAF. Fluorescent HMM failed to associate with myofibrils or any other recognizable structure when injected into living myotubes (*a*), even when alpha-actinin co-injected into the same cell (*b*) revealed the presence of well-developed myofibrils. Bar, 10  $\mu$ m.

A-bands. If this reflects the normal behavior of endogenous myosin, then one may conclude that myosin molecules, once incorporated into the thick filaments, are stably associated. This finding is consistent with the turnover rates determined for myosin in myofibrillar structures (Clark and Zak, 1981;

Rubinstein et al., 1976; Zak et al., 1977). Similar slow exchange for alpha-actinin molecules has also been observed along myofibrils in chick cardiac myocytes (McKenna et al., 1985). However, the slow rate does not appear consistent with the rapid incorporation of fluorescent myosin after mi-



**Figure 10.** Glycerinated myotubes incubated with IAF-labeled HMM in the absence and presence of ATP. When IAF-labeled HMM was added to glycerinated cells in the absence of ATP, bright fluorescence was detected in the region of the I-bands (*a*), as shown by comparison with the corresponding alpha-actinin image (*b*). However, when IAF-labeled HMM was added to glycerinated myotubes in the presence of MgATP, only diffuse fluorescence was observed (*c*), although the corresponding alpha-actinin image indicated the presence of well-developed myofibrils (*d*). Arrows, corresponding locations of the same Z-lines in each pair. Bar, 10  $\mu$ m.



**Figure 11.** Localization of IAF-labeled LMM microinjected into living myotubes. When IAF-labeled myosin was digested to yield LMM and injected into living myotubes (a), periodic fluorescent bands similar to those seen with whole myosin were visible. After glycerination of injected cells (b) and subsequent application of IATR-labeled alpha-actinin (c), it was evident that the fluorescent LMM was localized at the A-band. The slight decrease in sarcomeric length is due to a partial contraction that is common during glycerination. Arrows, corresponding locations of the same Z-lines in each pair. Bar, 10  $\mu$ m.

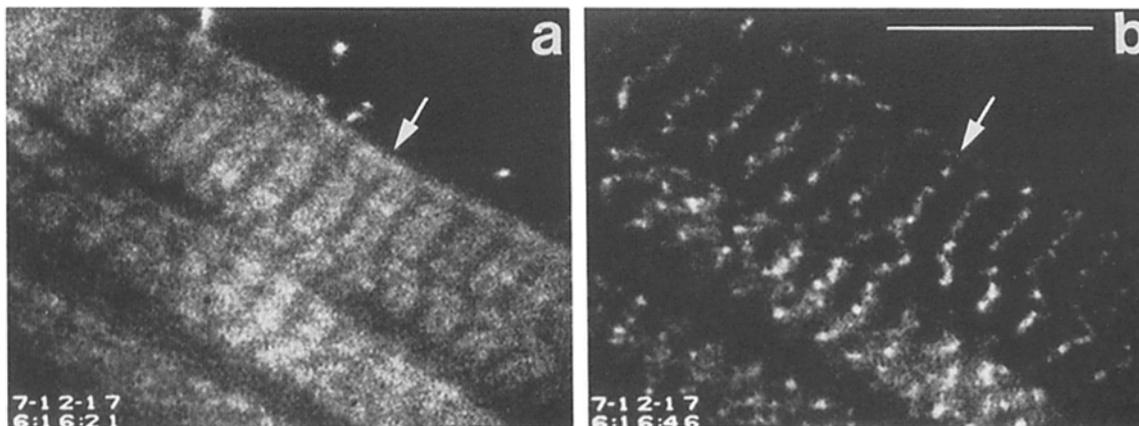
croinjection. One possible explanation is that the initial rapid incorporation may occur because of the availability of large numbers of unoccupied binding sites within the sarcomere, and/or the transient increase in the concentration of unassembled myosin molecules after microinjection. Once associated, the rate of dissociation may be very low. However, this slow exchange does not appear to be an inherent property of purified myosin, since a rapid exchange between a soluble myosin pool and polymerized synthetic filaments *in vitro* has been reported (Saad et al., 1986).

What are the roles of the different regions of myosin in the assembly of thick filaments? Several observations indicate that the head portion of the myosin molecule, which contains the enzymatic sites for actin-myosin interaction, does not have a substantial effect on the association of myosin with the A-band. First, fluorescent myosin was able to associate with the A-bands of glycerinated myofibrils in the presence of MgATP. If actin-myosin interactions were involved in this process, one would expect binding to be inhibited, similar to the lack of binding of labeled HMM under this condition. In addition, the LMM portion of myosin appeared to maintain the capacity to incorporate into the thick filament, whereas HMM injected into living cells was unable to incor-

porate into myofibrils, again indicating that the LMM portion is critical for the binding and that the HMM portion of myosin (S2 + heads) alone is insufficient.

However, the involvement of the head portion of myosin in some aspects of the assembly of thick filaments still cannot be discounted. As proposed by Maw and Rowe (1986), the initial formation of thick filaments may require the attachment of myosin heads to actin filaments in order to form a scaffold for the subsequent assembly. Moreover, the assembly of whole myosin into synthetic filaments appears to be modulated to some extent by MgATP (Chowrashi and Pepe, 1986; Pinset-Harstrom, 1985), whereas the rod was unable to respond to such modulation (Pinset-Harstrom, 1985). Therefore, although the MgATPase sites do not appear to play a major role in structural assembly, subtle influences from this region may be undetectable with the techniques used in this study.

The results of this study provide some insight into the behavior of myosin in living cells. Additional experiments using this approach should allow us to examine the role of myosin in both the development of muscle cells and the motility of nonmuscle cells.



**Figure 12.** Localization of IAF-labeled LMM in glycerinated myotubes. When IAF-labeled LMM was applied to previously glycerinated, uninjected myotubes, the conjugate again localized at the A-bands of myofibrils (a), as shown by the corresponding alpha-actinin image (b). Arrows, corresponding locations of the same Z-lines in each pair. Bar, 10  $\mu$ m.

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